The effects of neuregulin-1β on synthesis and secretion of surfactant phosphatidylcholine by cultured fetal rat type II pneumocytes.

by

George King BSc MSc

This thesis is presented for the degree of Doctor of Philosophy

at Murdoch University.

School of Veterinary and Life Sciences

Murdoch University

Western Australia

2013

No man can serve two Masters

Mathew: 6:24

Actions Speak Louder than Words.

Abraham Lincoln

Never too old to learn

Thomas Middleton

One crowded hour of glorious life is

worth an age without a name

Sir Walter Scott

Knowledge is power

Proverbs : 24:5

Little drops of water, little grains of sand,

make the mighty oceans and the pleasant land.

Julia Carney

Declaration

I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution.

George King

Abstract

In the latter stages of gestation the fetal lung undergoes biochemical maturation leading to the acquisition of the capacity to synthesize pulmonary surfactant. If insufficient amounts of surfactant are generated neonatal respiratory distress syndrome (NRDS) is likely to occur. The production of surfactant is known to involve a cellular communication between lung fibroblasts and the adjacent type II pneumocytes. Glucocorticoids have been shown to induce the production by lung fibroblasts of a soluble peptide, fibroblast-pneumocyte factor (FPF), which in turn acts on type II pneumocytes to enhance the synthesis of surfactant (Smith, 1978). Although Torday *et al.* (2002) have proposed that leptin has many of the characteristics of FPF, recent research has revealed that the peptide neuregulin-1β also has many of these characteristics (Dammann *et al.*, 2003).

Neuregulin was first purified from brain tissue and named glial growth factor (Brockes *et al.*, 1980). Subsequently, it was established that the neuregulins are a family of EGF-like growth factors that have been shown to be present in an increasing number of tissues and organisms. Neuregulin-1β stimulates both cell growth and differentiation in a range of target tissues by binding to ErbB3 and ErbB4 receptors thus stimulating the phosphorylation of tyrosine residues of the ErbB receptors and inducing receptor heterodimerization (Riese *et al.*, 1995; Carraway III *et al.*, 1997; Crovello *et al.*, 1998; Mautino *et al.*, 2004).

In this study, a commercially available form of neuregulin-1β (heregulin-1β) has been shown to directly stimulate synthesis of surfactant phospholipids in cultured fetal rat type II pneumocytes. Prior exposure of the type II cells to 20-50 $ng.mL^{-1}$ heregulin-1β for 21 hours led to a more than 3-fold increase in the rate of phospholipid synthesis ($p < 0.05$), a result similar to that previously observed in cells exposed to leptin (Torday *et al.*, 2002). In addition to this effect, 50 $ng.mL^{-1}$ heregulin-1β was also shown to directly increase the rate of secretion of surfactant phospholipids from type II cells by 2.4-fold ($p < 0.05$). Furthermore, exposure of type II cells to this peptide enhances by approximately 2-fold the β-AR activity and, as a consequence, elevates the rate of (—)-isoproterenol-induced surfactant phospholipid secretion to the same extent. Although heregulin-1β alone has no effect on β-AR gene expression in type II cells, the level of expression of this gene was synergistically enhanced when the cells were exposed to both dexamethasone and this peptide. Thus, overall, these findings not only support but considerably extend the concept promoted by Dammann *et al.* (2003) that neuregulin-1β plays an essential role in the differentiation and maturation of the lung in the later stages of gestation. Moreover, they suggest that neuregulin-1β is a significant component of FPF.

In summary, this study has shown that in type II cells neuregulin-1β enhances both surfactant phospholipid synthesis and secretion, elevates the level of β-adrenergic receptors and boosts the response of both male- and female- derived cells to β-agonists. It is therefore suggested that the administration of neuregulin-1β, in combination with antenatal glucocorticoid treatment, may provide an improved therapeutic mechanism of preventing respiratory distress syndrome of premature infants.

Acknowledgements

Science is of small value unless it provides not only for the welfare of life on earth, but also and more importantly for the recognition of the divine combination of truth. First praise is to Jesus Christ the Creator for giving me an insight into the complex yet perfect design of the cell.

My heartfelt thanks to my primary supervisor Emeritus Professor Max Cake, whose unwavering support and enthusiasm for this project have been inspirational. Whenever I was stuck, or had run out of ideas, he always had a new approach to ensure my research maintained momentum. In spite of his busyness, he always made time for a discussion of my work, and I consider myself fortunate to have worked with him. Furthermore, I will always remember his commitment to assist me in the preparation of this thesis during his retirement. Without this timely and generous assistance, this work would never have been completed, and I thank him for that. I am particularly pleased and honored to mention that my supervisor Professor Cake obtained his PhD in Biochemistry in 1974 under Professor Ivan T. Oliver the student of Sir Hans Adolf Krebs, the winner of the 1953 Nobel Prize in Medicine. I will be forever proud of having had Professor Cake as the principle supervisor of my PhD.

I am also very grateful to my second supervisor Dr David Berryman, whose expertise in PCR ensured successful completion of the molecular aspects of this study. He always managed to squeeze me in for a meeting, often at very short notice, which enabled me to establish and manage the PCR techniques.

Words cannot adequately express my gratitude to my third supervisor Dr Garth Maker. His previous research carried out in the same laboratory inspired me and his appointment to this role maintained continuity when Professor Cake moved into part-time employment. I also thank Dr Maker for his rapid response and critical suggestions for improvement of the draft versions of this thesis.

I cannot forget to mention my gratitude to Dr Lambert Brau, Dr James Haile and Dr Barbara Bowen for their generous offer of part-time employment, which gave me great experience in Cell Biology, Genetic Engineering and Environmental Biology, respectively. I look forward to continuing our collaboration in the years to come.

To my many research colleagues, I thank you all. Dr Mike Bunce, Dr Chris Florides, Mr Gordon Thomson, Dr Ian Wright, Dr Michael Calver, Ann Roberts, Frances Brigg and Ms Maria Waters were all supportive of me during my time at Murdoch University.

I would also like to thank Dr Jolanta Damas and Liong Ka Hang for their assistance and collaboration in the laboratory as well as granting permission to use some of their research findings in this thesis (Figures 3.23 and 2.10, respectively).

I want to thank Ms Cecily Scutt from the GREAT program for her advice on improving my writing skills, which really helped me in the organization and structuring of this thesis.

To my family, Maria, Sedra and Laura, I thank you for your support and understanding throughout this project. I could not have done it without this support. Finally to Rana, my beautiful and lovely wife, sorry it took so long! Thank you for your never-ending support, good humour and love. Without you, I can honestly say that I would never have even come close to reaching this point. Your endless enthusiasm was a true inspiration, and I am sure you do not realize just how much you helped me.

During the period of my research into lung development I enjoyed the inspiration I received from major contributors in the field and whose works are given credit in the thesis. I am honored and feel very privileged that my thesis was examined by Professor Heber Nielsen, Tufts University, Boston, MA, USA. Professor Martin Post, University of Toronto, Toronto, Ontario, Canada and Professor Mary Sunday, Duke University, Durham, NC, USA.

Financial support for this project was provided by the Commonwealth Government, through an Australian Postgraduate Award, and Murdoch University, through a Centre of Research Excellence scholarship. This support is gratefully acknowledged.

Table of contents

Index to figures

Index to tables

Abbreviations

β-AR	β-Adrenergic receptor
ACTB	Actin-β
ATP	Adenosine 5'-triphosphate
BSS	Balanced salts solution
CaCM-PK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	3,5-cyclic monophosphate
CCAAT-EBPα	CCAAT-enhancer binding protein alpha
CRD	Carbohydrate recognition domain
CREB	cAMP response element-binding protein
Ct	Cycle threshold
dpm	Disintegrations per minute
DPPC	Dipalmitoyl phosphatidylcholine
DSPC	Disaturated phosphatidylcholine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FCM	Fibroblast-conditioned medium

FPF	Fibroblast-pneumocyte factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGF	Glial growth factor
GPCRs	G protein-coupled receptors
GRP	Gastrin releasing peptide
IgG	Immunoglobulin G
LB	Lamellar body
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEM	Minimal Essential Medium
MEM^{+}	Minimal Essential Medium plus antibiotics
MEM^{C}	Charcoal-treated serum-supplemented MEM^{+}
MW	Molecular weight
mRNA	Messenger ribonucleic acid
NBCS	Newborn calf serum
NRDS	Neonatal respiratory distress syndrome
NRG	Neuregulin
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PG	Phosphatidylglycerol
Pgk	Phosphoglycerate kinase
PI	Phosphatidylinositol
PKA	Protein kinase A (cAMP-dependent protein kinase)
PKC	Protein kinase C
Ppia	Peptidylprolyl isomerase A
PTH	Parathyroid hormone

PTHrP	Parathyroid hormone-related protein
qPCR	Quantitative polymerase chain reaction
Rpl13a	Large ribosomal protein 13a
rpm	Revolutions per minute
siRNA	Small interfering RNA
SP	Surfactant protein
Tfrc	Transferin receptor
TGF-β	Transforming growth factor-β

Units

°C	degrees celsius
Ci	Curie
cm	centimetre
dpm	disintegrations per minute
g	gram
g	centrifugal force
kDa	kiloDalton
L	litre
M	moles.litre^{-1} (molar)
mg	milligram
mL	millilitre
µCi	microCurie
µg	microgram
µL	microlitre
mM	millimoles.litre^{-1} (millimolar)
µM	micromoles.litre^{-1} (micromolar)
µm	micrometre
ng	nanogram
nM	nanomoles.litre^{-1} (nanomolar)
pg	picogram
rpm	revolutions per minute
IU	international unit
V	volt

Publications arising from this thesis

- **King, G**., Cake, M. H., Berryman, D. and Maker, G. L (2013), *Influence of glucocorticoids, neuregulin-1β and sex on surfactant phospholipid secretion from type II cells*, in ComBio meeting, Perth Convention and Exhibition Centre, Australia 6000.

- **King, G**., Maker, G. L., Berryman, D., Trengove, R. D. and Cake, M. H (2013), *Role of neuregulin-1β in dexamethasone-enhanced surfactant synthesis in fetal type II cells*, in: Royal Society of Western Australia, Murdoch University, Murdoch, Australia 6150.

- **King, G**., Damas, J. E., Cake, M. H., Berryman, D. and Maker, G. L. (2014) Influence of glucocorticoids, neuregulin-1β and sex on surfactant phospholipid secretion from type II cells. Am. J. Physiol. Lung Cell Mol. Physiol. **306**: in press.

- **King, G**., Maker, G. L., Berryman, D., Trengove, R. D. and Cake, M. H. (2014) Role of neuregulin-1β in dexamethasone-enhanced surfactant synthesis in fetal type II cells. FEBS Lett., submitted.

.

Chapter 1

General introduction

1.1 General introduction

The alveolar surfaces of the lungs are lined with a heterogeneous material called pulmonary surfactant, which forms a monolayer on the internal surface of the alveoli. The main role of surfactant is to reduce the surface tension at the air: liquid interface. This prevents alveolar collapse at low pressures and allows the lung to remain filled with air even after expiration. The functional properties of surfactant are provided by its phospholipids and proteins, both of which are necessary for establishing and maintaining a low surface tension. Since the lungs are not involved in gas exchange until after birth, it is not surprising that surfactant is only formed in the later stages of development. Premature delivery of infants is often accompanied with lung immaturity, resulting in a deficiency of surfactant (Clements and Avery, 1998; Avery, 2000). This phenomenon leads to neonatal respiratory distress syndrome (NRDS), a condition that, if untreated, can be fatal.

Throughout gestation, the human fetal lung develops within its fluid intrauterine surroundings, but must adjust to the extra-uterine environment at birth. There are many physiological processes that must take place to allow this adjustment, and key among them is the induction of pulmonary surfactant. Pulmonary surfactant is a complex, surface-active material that contains phospholipids and the surfactant-associated proteins SP-A, SP-B, SP-C, and SP-D. Surfactant is known to generate a monolayer lining at the air: liquid interface of the lung (Griese, 1999) (Figure 1.1).

The air: liquid interface within the alveoli is subjected to large variations in the surface tension which, if unmodified, would lead to progressive atelectasis and ultimately respiratory collapse (Griese, 1999). The primary function of pulmonary surfactant is to reduce this surface tension to very low values, and dynamically adjust

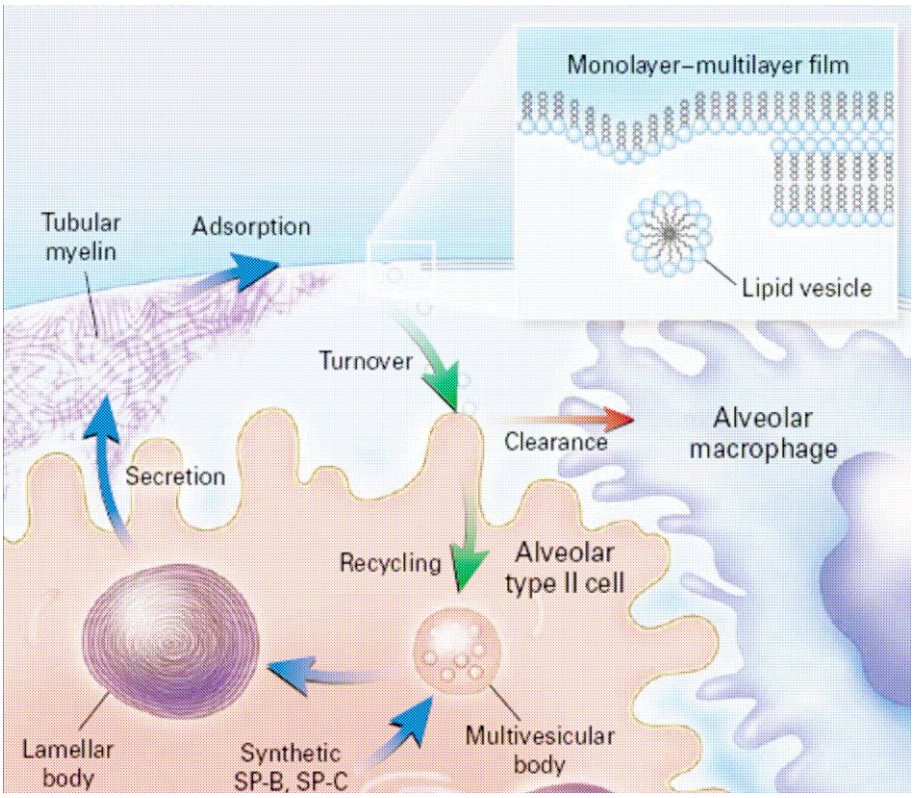

Figure 1.1 **Diagrammatic representation the alveolar space with the air: liquid interface magnified to illustrate the surfactant monolayer** (Whitsett and Weaver, 2002).

the tension as the surface area changes with each inspiration and expiration (Johannson and Curstedt, 1997). Surfactant also functions to protect the lung from injury and infection that may be caused by the inhalation of particles and microorganisms (Griese, 1999).

1.2 Lung maturation

Lung epithelial cell differentiation is dependent upon a mesenchymal-epithelial cell communication. The fetal lung undergoes an extensive morphological and biochemical maturation during late gestation to enable it to be capable of gas exchange. For example, there is a dramatic increase in the production of the highly surface-active substance known as pulmonary surfactant. Its main functional component is disaturated phosphatidylcholine (DSPC), which lowers surface tension and prevents alveolar collapse (Buckingham and Avery, 1962). Differentiation of the respiratory epithelial cells lining the lung increases in the later stages of lung morphogenesis. Lung maturation is associated with (i) the generation of lung saccules and the formation of septae to produce alveoli, (ii) synthesis and secretion of pulmonary surfactant required to reduce surface tension at the air: liquid interface after birth, (iii) clearance of lung fluids after birth, (iv) thinning of the alveolar walls, and (v) the development of the pulmonary vascular bed to facilitate the process of gas exchange across the alveolar capillary barrier (Besnard *et al.*, 2011).

1.3 History of the surfactant system

Research on surfactant began in the mid 20th century when Thannhauser *et al.* (1946) reported that lung tissue has an extraordinarily high content of the lipid dipalmitoyl lecithin (currently termed dipalmitoyl phosphatidylcholine). At that time, no connection was made between the high content of this lipid and stabilization of the alveoli. Nine years later, Pattle proposed that bubbles, made of a lung fluid substance,

obtained their steadiness through the quantity and quality of the surface-active material (Pattle, 1955).

Clements *et al.* (1958) showed, with the help of a modified surface balance, that surface tension dropped to low values upon compression of surface films generated using lung extracts. Clements and his group were also the first to investigate the surface tension-lowering properties of several lipid fractions and found that it was the phospholipid fraction that reduced surface tension. This effect was repressed by other lipid fractions (cholesterol, triacylglycerols, and fatty acids). Subsequently, it was reported that the activity of synthetic dipalmitoyl phosphatidylcholine (DPPC) was similar to that of the phospholipids isolated from fresh beef lung (Klaus *et al.*, 1961). Later it was shown that DPPC was produced by the developing lung and secreted into the alveolar space (Gluck *et al.*, 1967). Hallman and co-workers discovered the importance of phosphatidylglycerol (PG) in contributing to surfactant spreading and that children suffering from respiratory distress syndrome had diminished levels of this phospholipid (Hallman *et al.*, 1977). Even though PG contributes only 5-7% of the total weight of purified surfactant, it is highly enriched relative to its content in mammalian membranes and, in conjunction with surfactant proteins SP-A and SP-B, appears to be important in the formation of tubular myelin (Wright, 1997).

It was subsequently documented that lipid extracts alone were not sufficient to reduce surface tension and attention focused on the presence and role of the proteins in surfactant (King *et al.*, 1973). This resulted in a rapid expansion of research into the molecular biology, structure, and properties of pulmonary surfactant proteins. In 1989 a new nomenclature for surfactant proteins was proposed. The proteins were termed surfactant protein A, B, and C and subsequently a new protein, termed SP-D was added (Persson *et al.*, 1989) .

Although our understanding of the composition of surfactant and the structure of surfactant proteins has advanced greatly, the various functions of the surfactant proteins remain only partially understood. Nonetheless, artificial surfactants that include surfactant proteins have been used to treat neonates weighing 500-1500 g and suffering from respiratory distress syndrome. In a comparative study, infants were divided into two groups according to whether they were born before or after these surfactants were introduced into clinical practice. Regression models controlling for race, sex, and birth weight were used to assess the incidence of mortality. The odds of death for very low birth weight infants were shown to be reduced by 30 percent after exogenous surfactants were introduced (Schwartz *et al.*, 1994).

1.4 Anatomical aspects of the lung

The lung is a large organ (6 percent of the body volume, irrespective of body weight) with a large inner surface continuously in contact with the environment. Mammalian lungs are membranous sacs divided into alveoli, which are small sacs that vastly increase the surface area available for gas exchange. An analysis of the human lung indicates that 1 cm^3 of lung tissue has a total gas exchange surface of 300 cm^2. Because warm-blooded animals require a high rate of oxygen uptake, the large surface area is essential (Creuwels *et al.*, 1997). Although some gas exchange takes place in the terminal conducting airways, the alveoli are the principle site in which gas exchange occurs (Swenson *et al.*, 1992). The alveoli are bubble-shaped with a high curvature and are known to be the site where both oxygen and carbon dioxide diffuse across the capillary/alveoli interface (Bock *et al.*, 1929; Campbell, 1931).

The lung contains many different cell types (Copenhaver and Johnson, 1958; Brown and Longmore, 1981) but the production of pulmonary surfactant is regulated by the interaction between only two of these - epithelial type II pneumocytes

and mesenchymal fibroblasts (Smith, 1979). Smith found that a peptide, which he termed fibroblast-pneumocyte factor (FPF), is produced by the fetal lung fibroblasts in response to glucocorticoids. When fetal rats were injected on day 17 of gestation with 1 μg of this material, the rats showed on day 20, biochemical evidence of accelerated lung maturation when compared to littermate controls (Smith, 1979). Epithelial type II pneumocytes represent about 14.5% of the cell population of the lung parenchyma (Haies *et al.*, 1980). A feature of these cells is the presence of surfactant storage organelles called lamellar bodies that are surrounded by a limiting membrane and which fuse with the plasma membrane to allow secretion of their contents (Haagsman and Van Golde, 1991; Andreeva *et al.*, 2007). Type II cells synthesize surfactant components, which are then stored in the lamellar bodies until the appropriate stimuli trigger their contents to be secreted into the alveolar airspace. King and his coworkers reported that the contents of the lamellar bodies enlarge and upon secretion undergo complex alterations including formation of a lattice-like structure called tubular myelin (Wright, 1990). This process is triggered by calcium, as well as the environmental conditions within the alveolar space (King *et al.*, 1983; Schmitz and Muller, 1991).

Differentiation of type II pneumocytes is triggered by the subjacent mesenchyme (fibroblasts). Fibroblasts from the pseudo-glandular stage stimulate epithelial cell proliferation, whilst those from the saccular stage stimulate differentiation (Caniggia *et al.*, 1991). In addition, fibroblasts in close proximity to the epithelial cells produce mainly differentiation factors whilst those further away produce mainly proliferation factors (Deimling *et al.*, 2007). Pneumocyte cells do not respond to proliferation factors once they have developed beyond the saccular stage (Caniggia *et al.*, 1991). In the later stages of development the fibroblasts respond to glucocorticoids and secrete differentiation factors (Smith, 1979; Caniggia *et al.*, 1991). Glucocorticoids have been shown to bind more readily to fibroblasts adjacent to type II pneumocytes

than to those more distantly located, a consequence of the adjacent fibroblasts being enriched with glucocorticoid receptors (Caniggia *et al.*, 1991). A central role for glucocorticoids in lung differentiation and maturation has been demonstrated in corticotropin-releasing hormone-deficient mice, which die from respiratory insufficiency due to abnormal lung development, a result of reduced levels of glucocorticoids (Muglia *et al.*, 1995; Muglia *et al.*, 1999).

The use of whole lungs in studying cellular differentiation does not take into consideration the cellular variety found within lungs nor the contribution of the individual cell types (Douglas and Farrell, 1976; Dobbs, 1990). Post and his co-workers found that cultured fetal alveolar type II pneumocytes retain many of the characteristics of those cells *in vivo*, particularly their ability to synthesize and secrete phosphatidylcholine (PC). Cultured type II cells also retain the ability to proliferate and respond to fibroblast-pneumocyte factor (FPF) (Post *et al.*, 1984; Post and Smith, 1984). Several factors increase the quality of type II cell cultures: (i) when collagenase is used as the tissue disassociating agent the cultured cells have a higher purity, and (ii) the growth of these cells is optimised if the media is supplemented with fetal calf serum (Douglas and Farrell, 1976).

1.5 Components of pulmonary surfactant

The physiological value of surfactant was first recognised in premature infants with respiratory distress syndrome (also known as hyaline membrane disease) (Stefansson *et al.*, 2002), a condition which can now be treated by intra-amniotic administration of pulmonary surfactant (Zhang *et al.*, 2004). A range of other lung diseases are also associated with surfactant abnormalities (Griese, 1999). The latter author reported that pulmonary surfactant is a highly surface-active material consisting of both lipids and specific surfactant-associated proteins. Lung surfactant is synthesized

by epithelial type II pneumocytes and its component lipids and hydrophobic proteins (SP-B and SP-C) are stored in lamellar bodies and secreted by regulated exocytosis (Rooney, 2001). Surfactant plays a vital role in pulmonary physiology. It has the major biophysical function of preventing alveolar collapse and thus neonatal respiratory distress syndrome (Holm *et al.*, 1996; Johannson and Curstedt, 1997; Mallory Jr, 2001).

Biochemically, pulmonary surfactant is nearly nine-tenths lipids, comprising phospholipids, triglycerides, cholesterol and fatty acids (King, 1982; Johannson and Curstedt, 1997; Lang *et al.*, 2005), and phosphatidylglycerol (PG) (Rooney *et al.*, 1994). The majority of the pulmonary surfactant lipids are phospholipids and the most abundant phospholipid is phosphatidylcholine (PC). The PC component is largely disaturated phosphatidylcholine (DSPC), consisting mostly of dipalmitoyl phosphatidylcholine and palmitoyl-myristoyl phosphatidylcholine, and plays an essential role in decreasing surface tension (Bernhard *et al.*, 2011). Pulmonary surfactant also contains a relatively large portion of phosphatidylglycerol (PG) (Griese, 1999; Bernhard *et al.*, 2011). Studies on PG-deficient adult rabbits have shown no obvious changes to surfactant properties or function, although these studies did use phosphatidylinositol (PI) as a substitute for PG (Hallman *et al.*, 1985). Human infants born prior to the pre-natal increase in PG levels are more likely to suffer from respiratory distress than infants born after the increase (Hallman *et al.*, 1977), suggesting a definite role for phosphatidylglycerol in the function of surfactant. It is possible that the presence of phosphatidylglycerol helps with alveolar strength (Hallman and Gluck, 1976), or with the association of proteins with phospholipids (King and Martin, 1980).

Surfactant generally contains between 5-10% of protein (Figure 1.2). Surfactant-associated proteins (SP) are known to interact extensively with the phospholipids, including changing the structure and properties of the lipid layers and

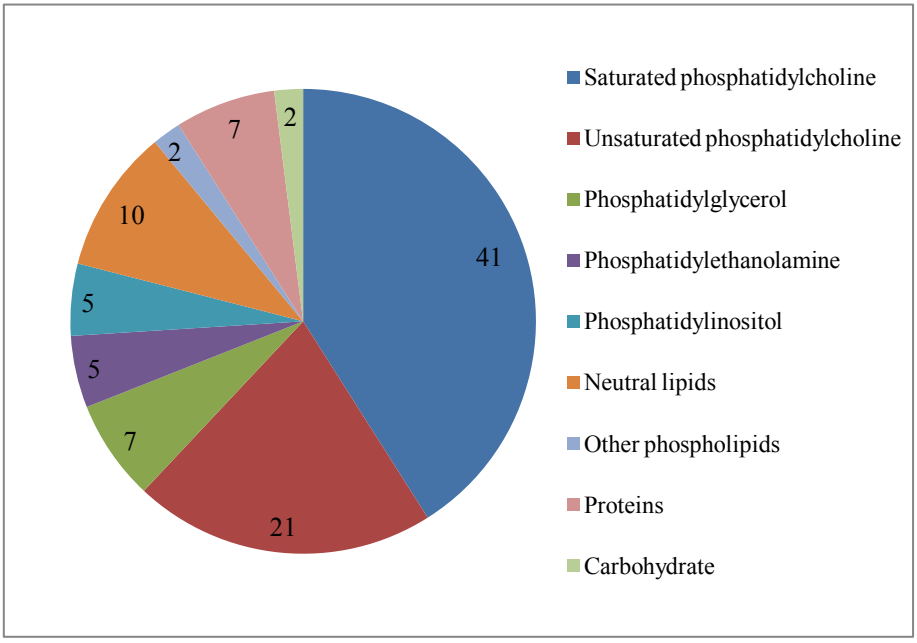

Figure 1.2 The composition of pulmonary surfactant expressed as a percentage by weight (Smith, 1979; King, 1982).

films (Yu and Possmayer, 1988; Dhand *et al.*, 1998). There are four surfactant proteins that have been identified: SP-A, SP-B, SP-C and SP-D. The general characteristics of each protein are summarized in Table 1.1.

Recent research has shown that the formation and stability of surfactant is highly dependent on surfactant protein B and C (Weaver and Conkright, 2001), while SP-A and SP-D are important components of the innate immune response to microbial challenge and participate in other aspects of immune and inflammatory regulation within the lung (Crouch and Wright, 2001). Type II pneumocytes secrete these proteins after exposure to leptin, which is produced and secreted by lung lipo-fibroblasts (Torday *et al.*, 2002). Leptin induces an increase in fetal lung weight in relation to the body weight due to an increase in the number of type II pneumocytes, which are responsible for producing SP-A, SP-B and SP-C proteins (Kirwin *et al.*, 2006). Thus, leptin appears to be a key cytokine regulator of fetal lung maturation. Other proteins have also been isolated with surfactant, including albumin and immunoglobulin, however, these may be contaminates that arise during tissue processing (King *et al.*, 1973). Chailley-Hey and her co workers (1999) found that vitamin A deficiency delays fetal lung maturation by reducing the expression of the gene for fatty acid synthesis. A deficiency of this vitamin also reduces the amount of mRNA coding for surfactant proteins A, B and C.

The most abundant surfactant protein by weight is SP-A. The SP-A monomer (molecular weight 26-36 kDa) is a glycoprotein with three separate structural domains (Johannson and Curstedt, 1997; Crouch, 1998). A collagen-like domain is connected via a linking region (a possible binding site for phospholipids) to a globular region, which contains a calcium-dependent carbohydrate recognition domain (CRD) that is able to bind both lipids, type II pneumocytes, and other structures, such as the

Table 1.1 Characteristics of the four surfactant-associated proteins, SP-A, SP-B, SP-C and SP-D (Yu and Possmayer, 1988).

Protein	Size	Structure	MW of polypeptide (kDa)	Polarity
SP-A	High	Octadecamer	26.0-36.0	Hydrophilic
SP-B	Low	Dimer	8.7	Hydrophobic
SP-C	Low	Monomer	3.5-5.0	Hydrophobic
SP-D	High	Dodecamer	39.0	Hydrophilic

surface of microorganisms (Griese, 1999). A complex oligosaccharide is also attached to this region of the SP-A molecule.

Because of their mixed collagen-like and globular structure, such molecules are called collectins. The fully processed and secreted form of SP-A consists of 18 SP-A monomers (octadecamer or six trimers) organized by means of covalent disulphide bridges and non-covalent interactions in the shape of a bunch of tulips (Griese, 1999). The two genes for human SP-A are both located on chromosome 10 and are expressed in alveolar type II pneumocytes, bronchiolar Clara cells and airway submucosal gland cells (Griese, 1999). Surfactant protein A and B both have a role in the conversion of endogenous surfactant into tubular myelin. Surfactant protein A accelerates the adsorption of surfactant phospholipids at the air: liquid interface, stimulates the defence system that depends on macrophages (Wright, 1997), reduces the inhibitory effect on surface activity of the non-surfactant proteins within the alveolar space, and possibly plays a role in the regulation of surfactant homeostasis (Ledford *et al.*, 2009).

Surfactant protein D is the second hydrophilic surfactant protein and is also a collectin (Wright, 1997). The collagen-like domain of SP-D is much larger than that of SP-A and is attached directly to the carbohydrate recognition domain without a connecting region. The molecular weight of the SP-D monomer is approximately 39 kDa. The native SP-D found in the lungs consists of 12 SP-D monomers, three of which are joined to form a trimer. Four trimers form a cross-shaped molecule. This cross-like structure (width of the molecule approximately 92 nm) may bind to bacterial lipopolysaccharides (LPS) and to cell surfaces, forming a large assembly of bacteria or cells (Griese, 1999). In addition, a receptor that binds SP-D, independent of its CRD domain, has been identified on alveolar macrophages (Holmskov *et al.*, 1997). SP-D is expressed in type II pneumocytes and in Clara cells; in humans the gene is located on

chromosome 10. The majority (70%) of SP-D is found dissolved in the watery surfactant residue, whereas SP-A, SP-B and SP-C are almost entirely found in association with lipids. SP-D is able to bind to phosphatidylinositol (Griese, 1999).

Surfactant protein C is the only surfactant protein that is exclusively expressed by type II pneumocytes in the mature lung. The human gene is found on chromosome 8; SP-C is translated as a larger precursor and is subjected to post-translational processing. The active molecule is a very hydrophobic polypeptide and two palmitoyl groups are attached via covalent bonds (molecular weight is approximately 4 kDa). The most important function of SP-C is to maintain the biophysical surface activity of the lipids. This occurs through an acceleration of the rate of adsorption at the air: liquid interface as well as through an increase in the resistance of surfactant to inhibition by serum proteins. SP-C stabilizes the surface activity of the surfactant film during the expansion and compression involved in breathing (Griese, 1999). Surfactant protein B and C also increase the uptake of phospholipids into type II pneumocytes.

Intra-alveolar surfactant protein B is a hydrophobic, positively charged molecule with a molecular weight of 8.7 kDa. Human SP-B is coded by a gene on chromosome 2 and is expressed in type II pneumocytes and Clara cells. SP-B is shaped mainly in the form of a dimer in the alveolar space, with the two SP-B molecules connected to each other via disulphide bonds. The main function of SP-B is to speed up the formation of a surface active film composed of phospholipids at the air: liquid interface by means of an increase in the phospholipid adsorption rate. This effect is further accelerated by the presence of calcium ions. SP-B in conjunction with SP-A and calcium ions is also involved in the formation of tubular myelin (Griese, 1999).

Type II cells can internalize surfactant components to be either degraded or recycled, thus suggesting that type II cells may serve both as a source of newly synthesized surfactant as well as a vehicle for clearance and recycling. It is reasoned that it is more economical in terms of cellular energy to recycle surfactant than to synthesise new material (Wright, 1990).

1.6 Neonatal respiratory distress syndrome (NRDS)

Neonatal respiratory distress syndrome, also known as hyaline membrane disease (Hallman and Haataja, 2007), is caused by alveolar collapse due to a deficiency of pulmonary surfactant (Ridsdale and Post, 2004; Zhang *et al.*, 2004). The surface tension of the moist inner surface, which originates from molecular attraction within a fluid, has the potential to cause collapse of the lung. This tendency is minimized by the presence of surfactant that reduces the surface tension at the inner surface of the alveoli to a very low level. Evidence suggests that surfactant is needed in the alveolar region of the lung as well as in the respiratory bronchioles, which conduct air to the alveoli (Liu *et al.*, 1991). *In vitro* studies have shown that a deficiency of surfactant leads to closure of these small cylindrical airways. In addition to this, the presence of proteases, and exuded plasma proteins in inflamed airways may harshly disrupt the functional ability of surfactant to keep the conducting airways open (Enhorning and Holm, 1993). Because of their small diameter, their ability to remain open is of particular importance to the physiological function of the alveoli. In areas with a relatively high surface tension (such as the alveoli) a thicker fluid film may build up. Thus, a well-functioning surfactant keeps the alveoli clear of liquid while also maintaining a thin surface film. A lack of surfactant, on the other hand, leads to the accumulation of oedematous fluid in the airspace. In general, pulmonary surfactant is believed to play a role in the physical removal of particulate material from the alveoli and small airways by means of the

displacement of particles into the hypo-phase and an improvement in the mucociliary clearance (Griese, 1999).

Under normal conditions, abundant phospholipid components (phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol) have been shown to suppress various lymphocyte and macrophage immune functions, whereas SP-A and SP-D have been confirmed to activate several immune cell functions (Wright, 1997; Crouch, 1998). Glasser *et al.* (2001) noted that SP-C is essential for stabilization of surfactant at low lung volumes. Although antenatal glucocorticoid administration to the mother prior to delivery can significantly reduce the incidence of NRDS, intratracheal administration of pulmonary surfactant to preterm infants greatly reduces neonatal morbidity and mortality from NRDS (Jobe, 1993).

In a recent study, Kevill *et al.* (2008) demonstrated that macrophage migration inhibitory factor (MIF) has a significant role in lung maturation during those stages of gestation when neonates are more susceptible to develop NRDS. MIF is expressed in bronchial epithelial cells and its concentration increases during gestation and subsequently declines to adult levels. Kevill and her coworkers found that higher intrapulmonary MIF levels were associated with lower likelihood of developing bronchopulmonary dysplasia, a result of NRDS.

Hallman and Haataja (2007) have stated that NRDS is a developmental disease caused by a number of factors. In addition to being induced by a transient deficiency of surfactant phospholipids, NRDS has been shown to be associated with allelic variants of the genes coding for the surfactant-associated proteins, SP-A, SP-B and SP-C. Furthermore, the preferential expression in type II cells of genes such as the ATP-binding cassette transporter, ABCA3 and those involved with known susceptibility to other severe lung diseases such as G protein-coupled receptor for asthma

susceptibility could also contribute to the development of NRDS (Hallman and Haataja, 2007).

1.7 Surfactant phospholipid synthesis

Alveolar type II cells, responding to various other factors produced by accessory cells (such as lung fibroblasts) are the major site of surfactant phospholipid synthesis (Smith, 1978; Torday *et al.*, 2002). Type II cells contain unique subcellular organelles called lamellar bodies consisting of many lamellae, which contain phospholipid bilayers, an electron dense core, and a surrounding limiting membrane (Snyder *et al.*, 1981; Stahlman *et al.*, 2000; Ridsdale and Post, 2004; Kirwin *et al.*, 2006). It has been shown that in late gestation undifferentiated, glycogen-laden type II cells change their metabolic program in the direction of synthesis of surfactant (Ridsdale and Post, 2004). The assembly of surfactant in these cells is not only derived from newly-synthesized components, but also from components taken up by the type II cells via endocytosis thereby facilitating recycling of the surfactant components (Haagsman and Van Golde, 1991; Andreeva *et al.*, 2007).

1.8 Surfactant phospholipid secretion

It is been proposed that direct stretching of type II pneumocytes during breathing is a physical stimulant to surfactant secretion (Lawson *et al.*, 1979; Copland *et al.*, 2006). Mechanical distortion of the cells resulted in a transient increase in the intracellular Ca^{2+} concentration, which triggered an increased rate of surfactant secretion that persisted for up to 30 minutes (Wirtz and Dobbs, 1990; Copland and Post, 2007). This delivery of surfactant to the alveolar space is preceded by this synthesis of the surfactant components followed by them being packaged into the subcellular lamellar bodies. Upon the receipt of an appropriate signal, the lamellar bodies accumulate adjacent to the apical plasma membrane where they fuse with the membrane

thus releasing the lamellar body contents. Secreted surfactant subsequently undergoes reuptake via endocytosis, intracellular processing and accumulation in lamellar bodies together with newly synthesized surfactant material. Therefore, intracellular surfactant is assembled from both newly synthesized and recycled surfactant components (Haagsman and Van Golde, 1991; Andreeva *et al.*, 2007).

1.9 Agents known to affect surfactant phospholipid synthesis

A wide range of hormones and other factors have been shown to influence the extent of surfactant phospholipid synthesis. In addition to glucocorticoids (which is discussed below), androgens, estrogens, growth factors (such as EGF and TGF-β), insulin, leptin, neuregulin-1β, prolactin and thyroid hormones have all been shown to influence the rate of surfactant phospholipid synthesis. In this chapter only some of the major factors will be discussed.

1.9.1 Glucocorticoids

Since the pioneering work of Liggins (1969), who demonstrated that the administration of dexamethasone to fetal sheep accelerated the appearance of lung surfactant, a number of investigators have shown that glucocorticoids enhance the production of surfactant phospholipids in a variety of species, including the rabbit (Kotas and Avery, 1971), mouse (Brehier and Rooney, 1981) and rat (Smith and Sabry, 1983). However, Dammann *et al*. (2006) have shown that this effect of glucocorticoids is influenced by the stage of development when administered to foetal mice. They demonstrated that whereas dexamethasone inhibited surfactant synthesis in immature type II pneumocytes at day 16 of gestation, it stimulated surfactant synthesis in the same cell type at day 17 of gestation.

As glucocorticoids are lipophilic their major mechanism of action involve them entering the target cell by diffusion across the cell membrane where they bind to

receptors located within the cell, as shown in Figure 1.3. An alternative, but less common, mechanism results from them binding to membrane-bound receptors. All the evidence (Post *et al.*, 1986; Sen and Cake, 1991; Alangari, 2010) suggests that the effect of glucocorticoids in promoting surfactant phospholipid synthesis is the result of genomic interactions.

This stimulatory effect is the result of an indirect action of glucocorticoids on lung fibroblasts to induce the production of a peptide, termed fibroblast-pneumocyte factor (FPF), which then acts upon the neighbouring type II cells to stimulate surfactant phospholipid synthesis (Post *et al.*, 1984; Post *et al.*, 1986) and surfactant protein A and B mRNA (Samtani *et al.*, 2006) . Smith and Fletcher (1979) observed that fetal lung pulmonary surfactant synthesis was dependent upon a molecular communication between mesenchymal cells and the neighbouring type II epithelial cells during pulmonary organogenesis. In particular, fibroblast-pneumocyte factor (FPF) was shown to be produced by the fetal lung fibroblasts in response to injecting fetal rats with 1 μg of glucocorticoids on the seventeenth day of gestation. Biochemical evidence of accelerated lung maturation was evident three days later. FPF, with a molecular weight of 5-15 kDa, is a heat stable, dialyzable polypeptide, which stimulates the biochemical activities of type II pneumocytes to synthesize and secrete pulmonary surfactant (Smith and Post, 1989).

1.9.2 Neuregulin

The neuregulins are a family of EGF-like growth factors that have been shown to be present in an increasing number of tissues and organisms (Carraway III *et al.*, 1997; Zhao *et al.*, 1998; Falls, 2003). Neuregulin, originally termed glial growth factor, was first purified from brain tissue (Brockes *et al.*, 1980). In other mammalian tissues it was

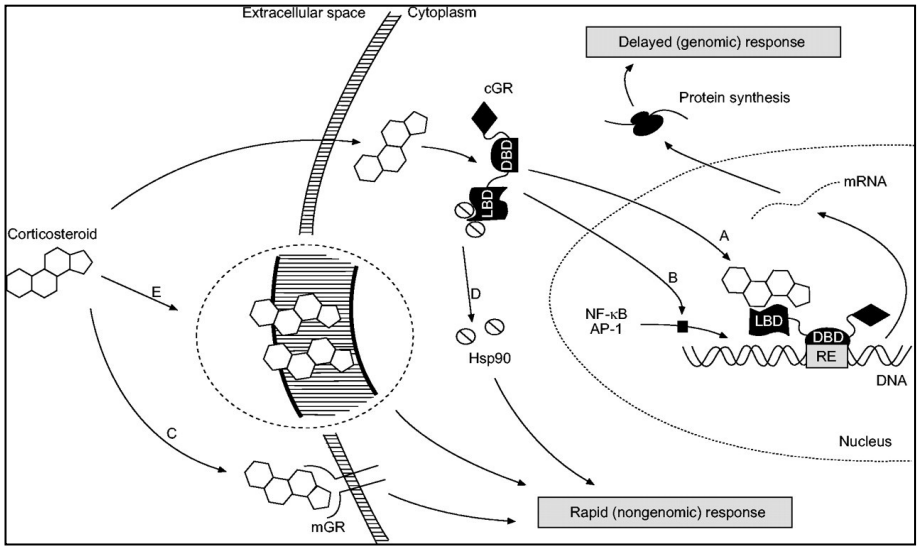

Figure 1.3 Mechanisms by which the actions of glucocorticoids are mediated.

(A) Direct DNA binding or (B) transcription factor inactivation. Non-genomic actions are mediated by (C) membrane-bound receptors, (D) cytosolic receptors or (E) interaction with cell membrane. cGR, cytosolic glucocorticoid receptor; mGR, membrane-bound glucocorticoid receptor; LBD, ligand-binding domain; DBD, DNA-binding domain; hsp 90, heat-shock protein 90; RE, response element; NFkP, nuclear factor k P; AP-1, activating protein-1 (Alangari, 2010).

named acetylcholine receptor-inducing activity (Falls *et al.*, 1990), neuregulin differentiation factor (Peles *et al.*, 1992) and heregulin (Holmes *et al.*, 1992). It was not until the 1990s (Marchionni *et al.*, 1993) that all of these different compounds were ascertained as the same protein and given the consensus name neuregulin, now known as neuregulin-1. This is because there are a number of neuregulins, many of which differ significantly in molecular weight and have a great variety of activities (Lemke, 1996). Thus, neuregulins belong to a multi-protein family of epidermal growth factor-like factors that arise from splice variations of a single gene (Carraway III *et al.*, 1997). The isoforms of neuregulin-1 are all generated from a single gene (Lemke, 1996), however, three other genes encoding neuregulin have also been discovered: neuregulin-2, 3 and 4 (Busfield *et al.*, 1997; Zhang *et al.*, 1997; Harari *et al.*, 1999). Little is known about the function of the proteins encoded by these three genes, although they are known to bind to the same receptors as neuregulin-1 (Falls, 2003) . Neuregulin-2 has been shown to elicit different responses to neuregulin-1 when binding to the same receptor (Crovello *et al.*, 1998). Unless otherwise stated, this review will focus solely on neuregulin-1.

Dammann *et al.* (2003) have shown that the stimulation of surfactant synthesis in type II cells by media, previously conditioned by fibroblasts in the presence of dexamethasone, can be mimicked by neuregulin-1β and inhibited by antibodies raised against this peptide. Thus neuregulin-1β plays a major role in the development and maturation of the fetal lung, preparing the lung for its function as an organ for gas exchange. These authors suggested that neuregulin-1β is a growth factor involved in many different stages of human development. Furthermore, Maker (2008) has shown that exposure of lung fibroblasts to dexamethasone leads to elevated levels of neuregulin-1β in the conditioned media. It is therefore possible that FPF may actually

be neuregulin-1β and its action involves interplay between neuregulin-1β and its specific receptors.

Neuregulin-1β has been shown to stimulate both cell growth and differentiation of target tissues by binding to ErbB3 and ErbB4 receptors and stimulates the tyrosine phosphorylation of other ErbB receptors through receptor heterodimerization (Riese *et al.*, 1995; Carraway III *et al.*, 1997; Crovello *et al.*, 1998; Mautino *et al.*, 2004). This ligand-induced heterodimerization is triggered by the bivalent nature of the ErbB ligands, a property which determines the specificity of dimerization. Of all these subtypes, the ErbB2 appears to be of particular importance as it is capable of forming dimers with ErbB1, ErbB3 and ErbB4. Structural studies have revealed that ErbB2 has a conformation which makes it constitutively available for dimerization (Cho *et al.*, 2003; Citri *et al.*, 2003) (Figure 1.4).

More recently, Zscheppang *et al.* (2007) and Liu *et al.* (2009) used a small interfering RNA (siRNA) against the ErbB4 gene to shut off ErbB4 receptor function in cultures of primary day 19 fetal rat lung type II cells. They showed that ErbB4-siRNA treatment not only diminished ErbB4 receptor protein expression, but also inhibited fibroblast-conditioned media (FCM)-induced ErbB4 phosphorylation, proliferation of the type II cells and the onset of fetal surfactant phospholipid synthesis through activation of the ErbB4 receptor. On the basis of a subsequent study it was concluded that deletion of pulmonary ErbB4 delayed both structural and functional aspects of fetal lung development, implying a crucial role for ErbB4 in the timely progression of fetal lung development (Liu *et al.*, 2010).

In human airway epithelia neuregulin is released from the apical membrane of the type II pneumocytes and is physically separated from ErbB2, ErbB3 and ErbB4 receptors, which are all located in the basolateral membrane. However, neuregulin finds

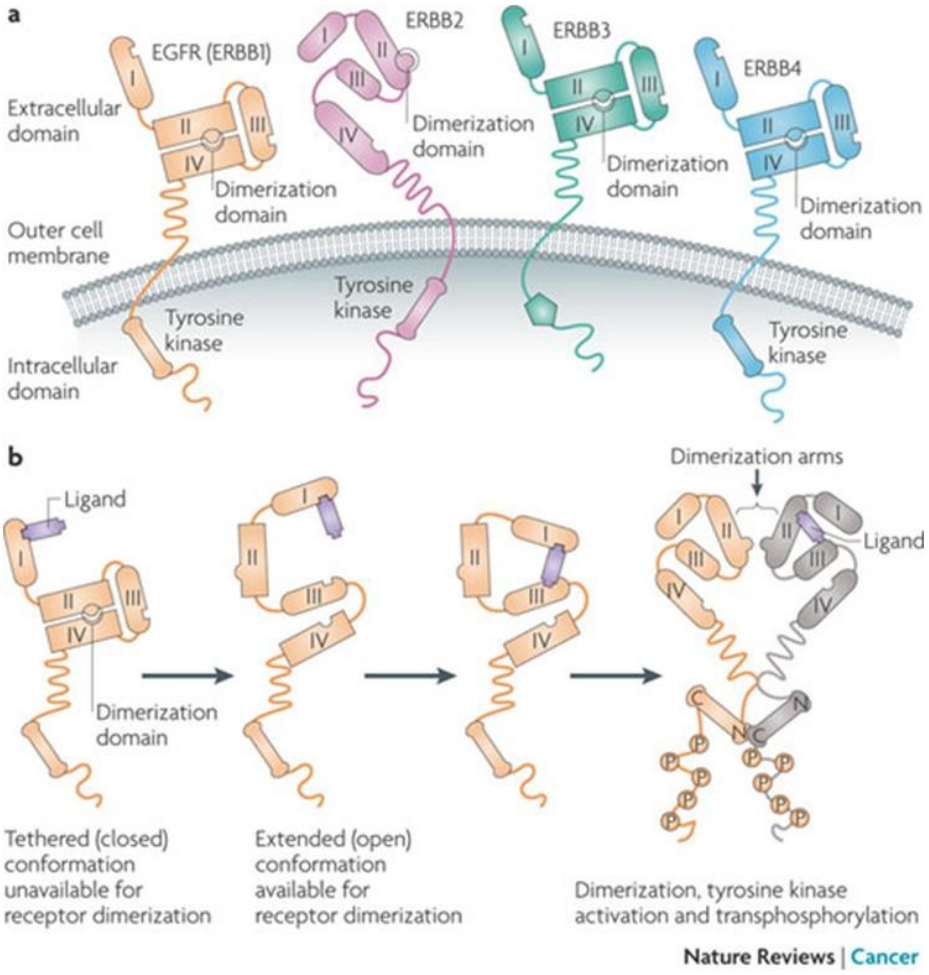

Figure 1.4 Structural conformation of the ErbB receptor family.

(A) structural conformations of individual members of the ErbB receptor family (B) ErbB is made up of back-to-back dimer, such that a dimerization arm from domain II of one subtype binds to a docking site at the base of domain II within the second subtype (Baselga and Swain, 2009).

its way to the basolateral part of the type II cells and creates a ligand-receptor pair poised for activation whenever epithelial integrity is disrupted by injury (Vermeer *et al.*, 2003).

1.9.3 Leptin

Leptin is a protein of 167 amino acids that is secreted by adipocytes and is essential in the regulation of energy balance within organisms (Hoggard *et al.*, 1997). It is a 16 kD hormone that participates in metabolic regulation, and also has role in inflammatory and immune reactions (Bruno *et al.*, 2009). This is evident from the observation that there is a decreased expression of leptin in the bronchial epithelium of untreated asthmatics (Bruno *et al.*, 2009). Hoggard *et al.* (1997) have shown that there is a high level of expression of leptin and its receptor in the lung of the 14.5-day mouse fetus suggesting that leptin may have a role in growth and development of this tissue.

Torday *et al.* (2002) and Kirwin *et al.* (2006) have independently shown that during rat lung development the expression of leptin by lipofibroblasts begins on embryonic day 17 and increases 7-10 fold by day 20. Leptin and its receptor are expressed exclusively by fetal lung fibroblasts and type II cells, respectively, which suggest the existence of a paracrine signaling loop. Using this mechanism, leptin stimulates the synthesis of surfactant phospholipid in both fetal rat type II cells and adult human airway epithelial cells. Leptin is secreted by lipofibroblasts in amounts that stimulate type II cell surfactant phospholipid synthesis *in vitro*. The synthesis and secretion of leptin by lung lipofibroblasts has been shown to be significantly enhanced when the cells are exposed to dexamethasone for 24 hours (Torday *et al.*, 2002). Such an observation is consistent with findings of Slieker *et al.* (1995) who showed that glucocorticoids increased both the levels of leptin mRNA and secretion of leptin from adipocytes.

The expression of parathyroid hormone-related protein (PTHrP) and PTH/PTHrP receptor are developmentally regulated in lung epithelial and mesenchymal cells, respectively (Rubin *et al.*, 2004). These authors investigated mice in which the gene encoding PTHrP was removed by homologous recombination using both *in vivo* and *in vitro* models. They found that fetal and newborn mice experienced delayed mesenchymal-epithelial interactions, arrested type II cell differentiation, reduced lamellar body formation and diminished pulmonary surfactant production. This was confirmed by Torday and Rehan (2006) who showed that PTHrP has its effect via increased levels of leptin. More recently it has been shown that during lung development in the *Xenopus laevis* tadpole leptin increased not only lung surfactant synthesis but also the size of the lamellar bodies and the diameter of the alveoli (Torday *et al.*, 2009).

Leung *et al.* (2006) demonstrated that glucose up-regulated not only the expression of leptin receptor in human peritoneal mesothelial cells but also the expression of leptin by adipocytes. By stimulating the level of leptin receptor, glucose is able to enhance the leptin-induced synthesis of TGF-β in these mesothelial cells. This latter process results from the activation of the Janus kinase-signal transducers and activation (JAK-STAT) signal transduction pathway.

1.9.4 Transforming growth factor-β (TGF-β)

While Dammann and Nielsen (1998) found that TGF-β increases EGF-R binding in lung fibroblast cultures from late gestation fetuses, Torday and Kourembanas (1990) demonstrated that TGF-β inhibits the development of lung cell maturation at specific gestational stages by blocking type II pneumocyte maturation. Moreover, McDevitt *et al.* (2007) have shown that endogenous TGF-β not only inhibits early morphogenesis but also blocks glucocorticoid-induced type II cell differentiation. As

these later authors found that dexamethasone reduced TGF-β bioactivity by decreasing TGF-β gene expression, they concluded that endogenous TGF-β suppresses normal lung development and proposed that glucocorticoid-induced type II cell differentiation is likely to involve attenuation of this suppression by TGF-β.

When one takes into account the influence of all of the afore-mentioned factors on surfactant synthesis it suggests that FPF, which stimulates the synthesis of surfactant in type II cells, could be NRG, leptin, PTHrP, or a combination of them all. Alternatively, glucocorticoids could stimulate type II cell differentiation by reducing synthesis of TGF-β in the fibroblasts and thereby attenuating the inhibitory effect of TGF-β (McDevitt *et al.*, 2007).

1.9.5 Other agents

A number of other studies have shown that surfactant phospholipid synthesis is induced by EGF and gastrin-releasing peptide (GRP) (Sunday *et al.*, 1990; Fraslon and Bourbon, 1992; Emanuel *et al.*, 1999), keratinocyte growth factor (KGF) (Xu *et al.*, 1998; Chelly *et al.*, 1999; Geshe *et al.*, 2011) and retinoids (Fraslon and Bourbon, 1994). KGF, otherwise known as fibroblast growth factor 7 (FGF-7), is a product of lung mesenchymal cells and has been shown to stimulate both the synthesis of the major component of surfactant, namely disaturated phosphatidylcholine, and the expression of the SP-A, -B and –C genes in a dose-dependent manner (Chelly *et al.*, 1999).

1.10 Agents known to affect surfactant phospholipid secretion

Although Dobbs and Mason (1979) were not clear on what factors controlled the secretion of pulmonary surfactant from alveolar type II pneumocytes *in vivo*, with the use of cultured cells they were able to verify that the β-adrenergic agonist (–)-isoproterenol stimulates surfactant secretion from type II cells. Other investigators

have shown that epidermal growth factor (EGF) (Sen, 1991), gastrin releasing peptide (GRP) and bombesin (Shan *et al.*, 2004) also stimulate the rate of secretion of surfactant lipids from cultured fetal rat type II pneumocytes (Asokananthan and Cake, 1996). The latter authors found that there was no response to GRP in the presence of saturating concentrations of A23187 or phorbol 12-myristate 13-acetate, implying that the secretory response to GRP is through activation of Ca^{2+}/calmodulin-dependent protein kinase and/or protein kinase C and is independent of adenosine 3′,5′-cyclic monophosphate-dependent protein kinase.

In a review article, Rooney (2001) stated that surfactant secretion is mediated by three different signalling pathways involving protein kinase A (PKA), protein kinase C (PKC) or calcium/calmodulin-dependent protein kinase (CaCM-PK). These signalling pathways are activated by a variety of secretagogues, some of which may be physiological regulators. In addition to those mentioned above, adenosine 5'-triphosphate (ATP) has been shown to interact with P_{2y}-purinoceptors thereby inducing Ca^{2+} mobilization, which leads to secretion of surfactant from type II cells (Rice and Singleton, 1987; Dorn *et al.*, 1989). Activation of these protein kinases leads to phosphorylation of proteins, which act to increase surfactant secretion through exocytosis of the lamellar bodies (Rooney, 2001). The proposed signalling transduction pathways involved in the secretion process are shown in Figure 1.5.

1.10.1 β-Adrenergic agonists

β-Adrenergic agonists are thought to raise the rate of surfactant discharge by enhancing the production of the secondary messenger, cyclic AMP. Binding of β-agonists to their specific receptors activates adenylyl cyclase via a stimulatory G protein (Aksoy *et al.*, 2002). The resulting production of the second messenger, cAMP,

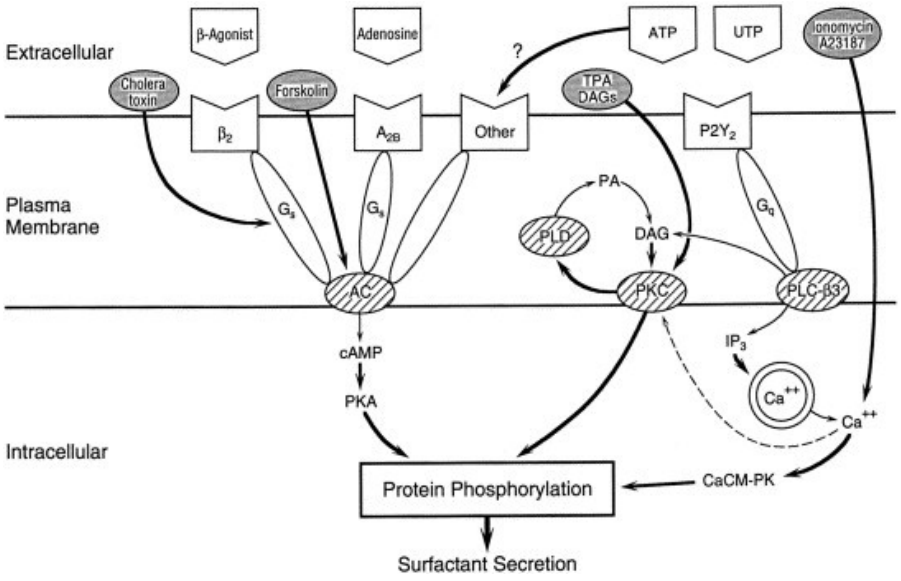

Figure 1.5 Signalling pathways leading to surfactant secretion (Rooney, 2001).

activates PKA leading to phosphorylation of cellular proteins. The increase in cAMP concentration, and its participation in motivating surfactant secretion via β-adrenergic receptor activation, has been implicated by the observation that (–)-isoproterenol causes a rise in the cAMP concentration in type II cells (Brown and Longmore, 1981).

The β-adrenergic receptor, which is located throughout the lung (Mutlu and Factor, 2008) and to which the β-agonists bind, is a member of a large subfamily of G protein-coupled receptors (GPCRs) that contain seven hydrophobic domains that span the cell membrane and are connected to each other by hydrophilic extracellular and intracellular loops (Strader *et al.*, 1995). Brown and Longmore (1981) have shown that the β-agonist-receptor interaction leads to the activation of a Gs protein, which results in an elevated level of cyclic AMP via the stimulation of adenylyl cyclase. This was confirmed by inhibition of cyclic-AMP production when an antagonist, alprenolol, was used in place of the agonist (Brown and Longmore, 1981). Moreover, Abraham *et al.* (2004) demonstrated a functional coupling between the β-AR level and the degree of activation of adenylyl cyclase in the cultured alveolar cell type A549.

Heterologous regulation of β-AR levels by glucocorticoids is a physiologically important example of how other factors can influence the regulatory processes exerted by β-adrenergic agents (Collins *et al.*, 1988; Hadcock and Malbon, 1988; Hadcock *et al.*, 1989). Numerous *in vitro* and *in vivo* studies have shown that the β-AR level and β-agonist-stimulated adenylyl cyclase activity are amplified by glucocorticoids (Cheng *et al.*, 1980; Giannopoulos and Sommers Smith, 1982; Maniscalco and Shapiro, 1983; Norris *et al.*, 1987; Collins *et al.*, 1988; Dangel *et al.*, 1996). The increase in β-AR numbers results from an increase in the rate of synthesis of new receptors (Norris *et al.*, 1987; Collins *et al.*, 1988), which is preceded by increased steady state levels of β-AR mRNA (Collins *et al.*, 1988; Mak *et al.*, 1995a; McGraw *et*

al., 1995; Cornett *et al.*, 1998; Aksoy *et al.*, 2002). Isoproterenol decreased β_1- and β_2-receptor numbers by 48 and 51% and also reduced mRNA expression by 69 and 57%, respectively. The combination of dexamethasone and isoproterenol did not change the level of β_2-receptors, but there was a major reduction of β_1-receptor and its mRNA expression. From these findings, Mak and his coworkers concluded that glucocorticoids can prevent homologous down regulation of β_2-receptor numbers and mRNA expression (Mak *et al.*, 1995b).

Recent findings have shown that the enhancement of the β-adrenergic response in type II cells by glucocorticoids is notably greater if the cells are exposed to culture media that has been conditioned by cultured lung fibroblasts in the presence of the steroid (Damas, 2010). This suggests that this effect of glucocorticoids may also involve fibroblast pneumocyte factor (FPF). Moreover, these latest findings have shown that there is a marked sex-linked difference in the stimulation of β-AR levels in response to glucocorticoids.

1.11 Effect of gender and gestational age on lung maturation

As described previously, surfactant deficiency is a major contributor to prenatal mortality in preterm infants. Miller & Futrakul (1968) demonstrated that NRDS occurs more often in male than in female infants, because lung maturation is less advanced in males (Torday *et al.*, 1981). Nielsen (1989) found that growth factors play a significant role in regulating the orderly progression of organ growth and differentiation during fetal development. He found that FPF is normally not produced by day 16 fibroblasts. In female-derived fibroblasts it initially appears on day 17 but in male derived fibroblasts its appearance does not occur until day 18. Exposure of the fibroblasts to EGF advanced this pattern by 24 hours such that female- and male-derived fibroblasts produced activity on day 16 and 17, respectively. By studying

two strains of mice with a gestational duration that differed by one day, Besnard *et al.* (2011) demonstrated that both fetal and maternal genotypes influences not only the timing of birth but also the extent of lung maturation at birth. Moreover, they identified an important role for the transcription factors CCAAT-enhancer binding protein alpha (CCAAT-EBPα) and cAMP response element-binding protein (CREB) in maternally induced lung maturation.

It is well established that surfactant appears earlier in the lungs of female fetuses (Nielsen and Torday, 1981; Torday *et al.*, 1981; Nielsen *et al.*, 1982; Torday, 1984). The earlier formation of surfactant in female neonatal lungs favors patency of small airways leading to a higher airflow and lower airway resistance compared with lungs of neonatal males. Androgens have been shown in a variety of species to inhibit fetal lung surfactant production by altering epidermal growth factor and transforming growth factor-β signalling events (Carey *et al.*, 2007b). In contrast, estrogens have been shown to exert a stimulatory effect, which is mediated by the α and β forms of the estrogen receptor (ERα and ERβ) in mice (Dammann *et al.*, 2000). In mice and rats adult females have more and smaller alveoli than their male counterparts, an attribute that is also mediated by ERα and ERβ (Massaro and Massaro, 2006; Carey *et al.*, 2007a).

As the gestational age at which pulmonary surfactant production is acquired is later in male fetuses, it is not surprising that this correlates with a higher incidence of NRDS in prematurely delivered males (Miller and Futrakul, 1968; Nielsen *et al.*, 1982; Provost *et al.*, 2009). More recently, it has been shown that there is also a sex-linked difference in the expression of *ApoA-1, ApoA-II, ApoC-II and ApoH* in fetal type II pneumocytes, with the expression of these genes being higher in cells derived from females (Provost *et al.*, 2009). They concluded that apolipoproteins had a role in lipid

metabolism and transport in the developing lung in association with the sex difference in synthesis of the surfactant lipids.

1.12 Research proposal

As discussed, Smith (1979) demonstrated that FPF was produced by the fetal lung fibroblasts in response to treatment with glucocorticoids and that this peptide was able to accelerate lung maturation through its ability to stimulate the synthesis and secretion of pulmonary surfactant in type II pneumocytes (Smith and Post, 1989). Since that time there have been numerous attempts to identify FPF. Torday and his coworkers (2002) found that during rat lung development, lipofibroblasts express leptin from a gestation age of 17 days. Leptin stimulates the synthesis of surfactant phospholipid in both fetal rat type II cells (Torday *et al.*, 2002; Kirwin *et al.*, 2006) and adult human airway epithelial cells (Torday *et al.*, 2002). Using semi-quantitative RT-PCR, it was shown that the expression of leptin was elevated when fetal rat lung fibroblasts were exposed to 10 nM of dexamethasone for 24 hours (Torday *et al.*, 2002).

Subsequently, Dammann *et al.* (2003) showed that the stimulatory effect of fibroblast conditioned media on surfactant synthesis by type II cells could be mimicked by exogenous neuregulin-1β and blocked by a neutralizing neuregulin-1β antibody. Moreover, both FCM and neuregulin-1β stimulated ErbB2 receptor phosphorylation in type II cells. More recently, it has been shown that exposure of cultured fetal lung fibroblasts to dexamethasone increases the concentration of neuregulin-1β in the FCM (Maker, 2008).

The above findings suggest that the main component of FPF, which stimulates type II cells to synthesize and secrete pulmonary surfactant, could be leptin, neuregulin or a combination of both (Figure 1.6). Although previous studies indicate that KGF is synthesized by mesothelial cells and stimulates the synthesis of surfactant

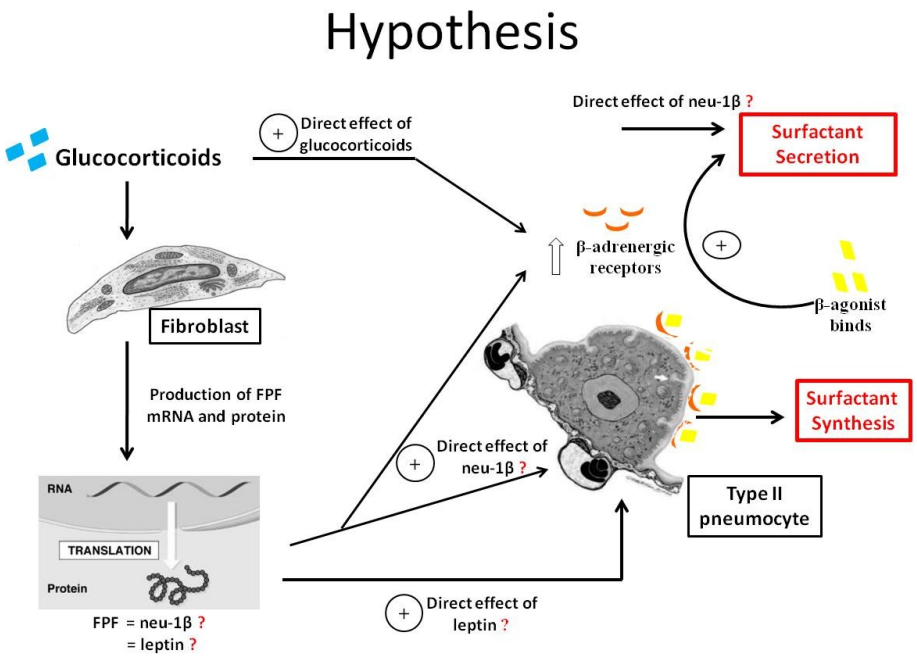

Figure 1.6 **Proposed actions of glucocorticoids, leptin and neuregulin-1β in the direct and indirect stimulation of surfactant phospholipids synthesis and secretion from type II pneumocytes.**

components, its size (28 kDa; (Rubin *et al.*, 1989) is much larger than the 5-15 kDa ascribed to FPF (Smith and Post, 1989). Thus, in this study the focus was on the specific effects of leptin and neuregulin-1β on surfactant phospholipid synthesis and secretion in cultured fetal type II pneumocytes. In particular, the specific aims were as follows:

- As the concentration of neuregulin-1β in fibroblast-conditioned media is elevated in response to glucocorticoids, the level of expression of neuregulin-1β will be used to determine whether or not glucocorticoids are able to up-regulate neuregulin-1β gene expression in cultured fetal rat lung fibroblasts.
- The impact of dexamethasone on the expression of this gene will be compared with that of TGF-β1, a gene known to be suppressed by this steroid (McDevitt *et al.*, 2007).
- To compare the time- and concentration-dependence of both neuregulin-1β and leptin stimulation of surfactant phosphatidylcholine synthesis in type II cells.
- To test the direct effect of leptin and neuregulin-1β on the secretion of surfactant phosphatidylcholine from cultured type II pneumocytes.
- To ascertain the effect of neuregulin-1β on the activity of the β-adrenergic receptor in type II cells and to examine this in conjunction with the ability of the secretagogue, (–)-isoproterenol, to stimulate surfactant phosphatidylcholine secretion.
- To determine if the secretory response of type II cells to neuregulin-1β is influenced by the sex of the donor animal.
- qPCR will be used to ascertain the direct effects of both glucocorticoids and neuregulin-1β, alone and in combination, on the expression of the β-AR gene in cultured type II pneumocytes.

Chapter 2

Regulation of surfactant phospholipid synthesis and secretion by neuregulin-1β, leptin and (–)-isoproterenol from cultured fetal type II pneumocytes

2.1 Introduction

Dexamethasone administration not only induced early delivery when injected into fetal lambs but also stimulated aeration of their lungs, which suggested that glucocorticoids may accelerate the appearance of surfactant (Liggins, 1969). A subsequent study showed that infants of women undergoing premature birth had a markedly reduced incidence of respiratory distress syndrome if the mothers were treated with glucocorticoids prior to delivery (Liggins and Howie, 1972). Further work has shown that glucocorticoids enhance the production of surfactant phospholipids in a variety of species, including the rabbit (Kotas and Avery, 1971), mouse (Brehier and Rooney, 1981) and rat (Smith and Sabry, 1983). Smith and colleagues have shown that the stimulation of lung maturation by glucocorticoids is an indirect effect involving a mesenchymal-epithelial cell interaction (Smith, 1978; Smith, 1979). They suggested that glucocorticoids stimulate lung fibroblasts to produce a peptide that they termed fibroblast-pneumocyte factor (FPF), which acts on the type II pneumocytes to enhance surfactant phospholipid synthesis (Post *et al.*, 1984; Post and Smith, 1984).

Torday and his colleagues have shown that, in response to being stretched, type II cells secrete parathyroid hormone-related protein (PTHrP), a product that stimulates pulmonary surfactant production by a paracrine feedback loop mediated by leptin, a soluble product of the mature fibroblast (Torday and Rehan, 2002). Furthermore, they have shown that leptin is released from fetal rat lung fibroblasts in response to dexamethasone exposure and that leptin, acting via specific receptors, is able to directly stimulate surfactant phospholipid synthesis in type II cells (Torday *et al.*, 2002). On the basis of these observations, they have suggested that leptin might be FPF. However, neuregulin-1β has also been shown to play a major role in the development and maturation of the fetal lung, preparing the lung for its function as an organ for gas exchange. Dammann *et al.* (2003) have provided evidence that the

stimulation of surfactant synthesis in type II cells by media, previously conditioned by fibroblasts in the presence of dexamethasone, can be mimicked by neuregulin-1β and inhibited by antibodies raised against this peptide. Moreover, Maker (2008) has shown that, by exposing lung fibroblasts to dexamethasone, the concentration of neuregulin-1β in the conditioned media was elevated by more than 2-fold. It is thus concluded that the 'FPF' response may actually be due to a combination of factors that includes both leptin and neuregulin-1β.

In this study, the focus of attention was heregulin-1β, a commercially available form of recombinant human neuregulin-1β. This peptide is a 7.5 kDa polypeptide consisting of the EGF-domain of human neuregulin-1β, which is known to exert its effect by binding to ErbB3 and ErbB4 receptors triggering their heterodimerization with ErbB2 and thus stimulating its associated tyrosine kinase (Crovello et al., 1998; Dammann et al., 2006). Activation of these receptors has been shown to result in differentiation of the lung (Dammann et al., 2003) and other tissues (Lemke, 1996; Burden and Yarden, 1997).

Exposure of mature (d18) mouse type II pneumocytes to dexamethasone has been shown to increase the expression of the receptors for neuregulin, namely the ErbB receptors, and also influence their pattern of dimerization (Dammann *et al.*, 2006). Neuregulin-1β and fibroblast-conditioned media have both been shown to elevate the levels of total and phosphorylated ErbB4 receptors (Zscheppang *et al.*, 2007; Liu *et al.*, 2009), as well as surfactant phospholipid synthesis and cell proliferation. These observations imply that ErbB4 receptors and, by inference, neuregulin-1β play a central role in facilitating the stimulation of surfactant phospholipid synthesis that is indirectly induced by glucocorticoids.

The secretion of surfactant phospholipids from type II cells is known to be regulated by β-adrenergic agonists (Dobbs and Mason, 1979; Brown and Longmore, 1981), which are released at the time of birth. Numerous *in vitro* and *in vivo* studies have shown that the β-AR level and β-agonist-stimulated adenylyl cyclase activity are amplified by glucocorticoids (Cheng *et al.*, 1980; Giannopoulos and Sommers Smith, 1982; Maniscalco and Shapiro, 1983; Norris *et al.*, 1987; Collins *et al.*, 1988; Dangel *et al.*, 1996). This has been shown to be the result of enhanced transcription of the β-AR gene (Mak *et al.*, 1995a; McGraw *et al.*, 1995; Aksoy *et al.*, 2002), a consequence of the β-AR gene containing a glucocorticoid response element (Cornett *et al.*, 1998). This heterologous regulation of the β-AR levels by glucocorticoids is a physiologically important example of how other factors can influence the regulatory processes exerted by β-adrenergic agents (Collins *et al.*, 1988; Hadcock and Malbon, 1988). Recent findings have shown that the response to glucocorticoids is noticeably greater if the type II cells are exposed to culture media that has been conditioned by cultured lung fibroblasts in the presence of the steroid (Damas, 2010), which suggests that this effect of glucocorticoids may also involve FPF. Moreover, these latest findings have shown that there is a marked sex-linked difference in the stimulation of β-AR levels in response to glucocorticoids.

In the current study the direct effects of neuregulin-1β on the rate of synthesis and secretion of surfactant phospholipids were examined in cultured fetal type II cells. The magnitude of these effects was also compared to those induced by leptin. In addition, the effects of neuregulin-1β on the type II cell β-AR level and the secretory response of these cells to (–)-isoproterenol were ascertained. The latter two investigations were carried out on type II pneumocytes that had been derived from either male or female rat fetal lungs.

2.2 Materials and methods

2.2.1 Animals

Nineteen-day pregnant rats of the Wistar strain of *Rattus norvegicus* were used for all experiments. The full gestation period of these animals is 22 days, so fetuses were delivered approximately three days premature. Animals were supplied by the Animal Resource Centre (Murdoch, Western Australia), whose mating protocol involves caging male and female rats overnight, followed by vaginal smearing the next morning. If sperm are detected conception is considered to have occurred. This is accurate to within eight hours of actual conception and is designated to be day zero. All experiments complied with the National Health and Medical Research Council guidelines and were approved by Murdoch University's Animal Experimentation Ethics Committee.

Sex of the fetuses was determined by differences in the external genitalia, which can be recognized as early as day 17 of gestation. The small urogenital orifice and the genital swellings are larger in males than in females. Also, the distance between the rectum and the urogenital sinus is greater in male rat fetuses (Nielsen and Torday, 1983).

2.2.2 Materials

Eagle's minimal essential medium (MEM) was obtained from Invitrogen Pty. Ltd., Mulgrave, Victoria, Australia. Newborn calf serum was obtained from Trace Biosciences Pty. Ltd., New South Wales, Australia. Penicillin G, streptomycin sulphate, amphotericin B from *Streptomyces sp* (Fungizone), activated charcoal, (–)-isoproterenol hydrochloride, immunoglobulin G (IgG), dipalmitoyl phosphatidylcholine (DPPC) and dexamethasone were supplied by Sigma-Aldrich, St. Louis, MO, USA. Filters (0.45 μm and 0.22 μm) were purchased from Advantec MFS Inc., CA, USA and Millipore Pty.

Ltd., North Ryde, Australia, respectively. Collagenase A (*Clostridium histolyticum*) was a product of Hoffman-La Roche Ltd., Basel, Switzerland. Ascorbic acid was obtained from May and Baker Australia Pty. Ltd, West Footscray, Victoria, Australia. PerkinElmer Inc., Boston, USA supplied [^{3}H-methyl]-choline chloride, L-α-phosphatidylcholine 1,2-[1-^{14}C]-dipalmitoyl and 1-[4,6-propyl-^{3}H]-dihydro-alprenolol. Tissue culture plates (60 cm; Falcon 35-3002), 60 cm Primaria culture plates (Falcon 35-3802), Primaria 24-well tissue culture plates and Optilux Petri dishes (Falcon 1001) were supplied by Becton Dickinson, North Ryde, New South Wales, Australia. Recombinant human neuregulin-1β (heregulin-1β) and recombinant rat leptin were obtained from PeproTech, Rocky Hill, NJ, USA. Whatman GF/A glass microfibre filters were supplied by GE Healthcare UK Ltd. Buckinghamshire, UK. Calcium chloride ($CaCl_2$), D-glucose, hydrochloric acid (HCl), magnesium chloride ($MgCl_2$), potassium chloride (KCl), sodium chloride (NaCl), sodium dihydrogen orthophosphate (NaH_2PO_4), disodium hydrogen orthophosphate (Na_2HPO_4), sodium hydrogen carbonate ($NaHCO_3$), were all of analytical reagent grade.

2.2.3 Preparation of materials for cell culture

2.2.3.1 Charcoal treatment of newborn calf serum

Newborn calf serum (NBCS) was incubated with sterile, acid-washed charcoal (50 mg.mL^{-1} serum) for 30 minutes in a 37°C shaking water bath to remove endogenous steroids. Charcoal was then removed by two successive centrifugations at 12096 *g* for 88 minutes at 4°C in a Beckman J2-21M/E centrifuge using a JA-20 rotor (Beckman Instruments, Palo Alto, CA, USA). Serum was filtered through a 0.45 μm Millipore GS filter to remove remaining charcoal, and then through a 0.22 μm Millipore filter to sterilize the serum. Charcoal-treated newborn calf serum was stored in 23 mL aliquots at -20°C.

2.2.3.2 Preparation of immunoglobin G (IgG) solution

An IgG solution was prepared by adding 100 mL of MilliQ filtered sterile water to 50 mg of purified IgG to yield a final concentration of 0.5 $mg.mL^{-1}$. Aliquots of 10 mL were then stored at -20°C. When required, IgG plates were prepared by dispensing 3 mL of the IgG solution onto Optilux bacteriological plates followed by air-drying of the dishes overnight at room temperature under ultraviolet light to maintain sterility. Prior to use, plates were washed 3 times with 10 mL of BSS.

2.2.3.3 Preparation of balanced salts solution (BSS)

Balanced salts solution (BSS) was prepared by mixing 900 mL MilliQ water, 50 mL 20x saline solution and 50 mL 20x buffer solution. The solution was adjusted to pH 7.4 and filtered through a 0.22 μm Millipore filter to sterilize. 20x saline solution contains 2.74 M NaCl, 54 mM KCl, 20 mM $CaCl_2$ and 20 mM $MgCl_2$. 20x buffer solution contains 119 mM $NaHCO_3$, 111 mM D-glucose, 27 mM Na_2HPO_4 and 3 mM NaH_2PO_4.

2.2.3.4 Preparation of amphotericin B

Amphotericin B solution was made by adding 10 mL of MilliQ water to 100 mg amphotericin B (Fungizone) and diluted with another 10 mL of MilliQ water to give a final concentration of 5 $mg.mL^{-1}$. Aliquots of 1 mL were stored at -20°C.

2.2.3.5 Preparation of culture media

Eagle's minimal essential media (MEM) was reconstituted as specified by the manufacturer, supplemented with 0.2% $NaHCO_3$ and the mixture adjusted to pH 7.4. Penicillin G and streptomycin sulphate were added to final concentrations of 0.0715 $g.L^{-1}$ and 0.1525 $g.L^{-1}$, respectively. Media were sterilized by filtration through a 0.22 μm Millipore filter and to each 208 mL an aliquot of amphotericin B (150 μL) was added. This solution is termed MEM^+, indicating it is MEM plus antibiotics. To produce

MEM complete (MEM^C), charcoal-treated, newborn calf serum was added to a concentration of 10%.

2.2.3.6 Preparation of DPPC solution

DPPC (12.5 mg) was dissolved in 5 mL of chloroform to yield a final concentration of 2.5 $mg.mL^{-1}$.

2.2.3.7 Preparation of [^{14}C]-labeled DPPC solution

Stock L-α-phosphatidylcholine 1,2-[1-^{14}C]-dipalmitoyl was diluted with ethanol such that a 50 µL aliquot yielded 10,000 dpm.

2.2.3.8 Preparation of heregulin-1β solutions

Lyophilized recombinant human neuregulin-1β (heregulin-1β; 50 µg) was dissolved in 500 µL MilliQ water and 25 µL aliquots dispensed into 1.5 ml microcentrifuge tubes, re-lyophilized and then stored at -20°C until use. Prior to use, heregulin-1β was redissolved in MilliQ water and appropriately diluted to yield the final concentrations referred to below.

2.2.3.9 Preparation of leptin solutions

Lyophilized recombinant rat leptin (200 µg) was dissolved in 1 mL MilliQ water and 25 µL aliquots dispensed into 1.5 ml microcentrifuge tubes, re-lyophilized and then stored at -20°C until use. Prior to use, leptin was redissolved in MilliQ water and appropriately diluted to yield the final concentrations referred to below.

2.2.4 Isolation of rat fetal lung cells

2.2.4.1 Isolation of lung fibroblasts

Sterile techniques were used during cell isolation procedures, all of which were performed at room temperature unless otherwise stated. Nineteen-day pregnant rats were killed by anaesthesia with 80:20% CO_2:O_2 exposure for 2 minutes followed by

asphyxiation with 100% CO_2 for another 2 minutes. Rats were sprayed with 70% ethanol prior to delivery of fetuses via Caesarean section. Fetal lungs were removed and placed in a Petri dish of warm BSS. Any other tissues, such as the heart and thymus, were removed and discarded. Lungs were briefly dried on a sterile napkin and then minced using a McIlwain tissue chopper (The Mickle Laboratory Engineering Co., Surrey, UK) set at 0.1 mm with two traverses, one perpendicular to the other. The minced tissue was then placed in a sterile flask with 0.75 mL warm BSS per lung containing collagenase A (0.247 $U.mg^{-1}$) at a concentration of 0.225 $mg.mL^{-1}$. The flask was then gassed with carbogen (95:5 O_2:CO_2) for 90 seconds to ensure sufficient oxygenation for cell survival, and incubated for 20 minutes in a 37°C shaking water bath.

Following incubation, the dissociated tissue was squeezed through two layers of sterile French *voile* using sterile forceps, into 30 mL centrifuge tubes. Additional warm BSS was added to ensure that all tissue was transferred from the culture flask to the tube. The mixture was centrifuged at 20 *g* at 4°C for two minutes in a Beckman GPR Centrifuge (Beckman Instruments, Palo Alto, CA, USA). The supernatant was removed and replaced with warm BSS, and the washing procedure repeated. MEM^+ solution was added so that the cells from two lungs were resuspended in each millilitre and plated onto 6 cm Falcon 35-3002 tissue culture plates (3 mL per plate), which were subsequently incubated for 30 minutes at 37°C in a humidified CO_2 incubator (5% CO_2 : 95% air) to allow adhesion of fibroblasts.

Following this incubation, non-adhering cells were removed from the plates after suspension by gentle swirling. To each of these tissue culture plates with the fibroblasts attached, 3 mL of serum-supplemented MEM^+ (MEM^C) was added, prior to incubation in a CO_2 incubator (5% CO_2 : 95% air) for further studies. With appropriate

changes of the media, plates are considered to be confluent after at least 8 days of incubation and consist predominantly of fibroblast cells (Figure 2.1)

2.2.4.2 Isolation of fetal type II pneumocytes

The non-adhering cells from the previously mentioned plates were transferred to an IgG-coated bacteriological plate (as described in 2.2.3.2). These plates were then incubated in a CO_2 incubator for an additional 1 hour. Unlike most lung cells, type II pneumocytes lack the F_c-receptor region (Dobbs *et al.*, 1986). This technique therefore allows lung cells, other than the type II cells, to bind to the IgG present on the plates, thus eliminating them from the cell mixture (Dobbs *et al.*, 1986). Following this incubation, the non-adherent cells were transferred to a sterile Shott bottle after gentle panning of the IgG plates, and charcoal-treated NBCS was added to a final concentration of 10%. Depending on the type of plate used and the number of lungs required per plate, the cell suspension was diluted using MEM^C. For phospholipid secretion experiments, a 0.5 mL aliquot was added to each 6 cm Primaria plate and incubated with 2.5 mL of MEM^C (equivalent to 1 lung/plate). For phospholipid synthesis experiments, a 0.25 mL aliquot was added to each 6 cm Primaria plate and incubated with 2.75 mL MEM^C (equivalent to 0.5 lung/plate). For the measurement of the β-adrenergic receptor level, 0.25 mL of diluted cell suspension was plated into each well of a Primaria 24-well tissue culture plate (6 lungs per 24-well plate). With appropriate changes of the media, the plates were considered to be confluent after 3 days and consisted predominantly of type II cells (Figure 2.2), as judged by Papanicolaou staining (Kikkawa and Yoneda, 1974) and electron microscopy of the cells (Maker, 2008).

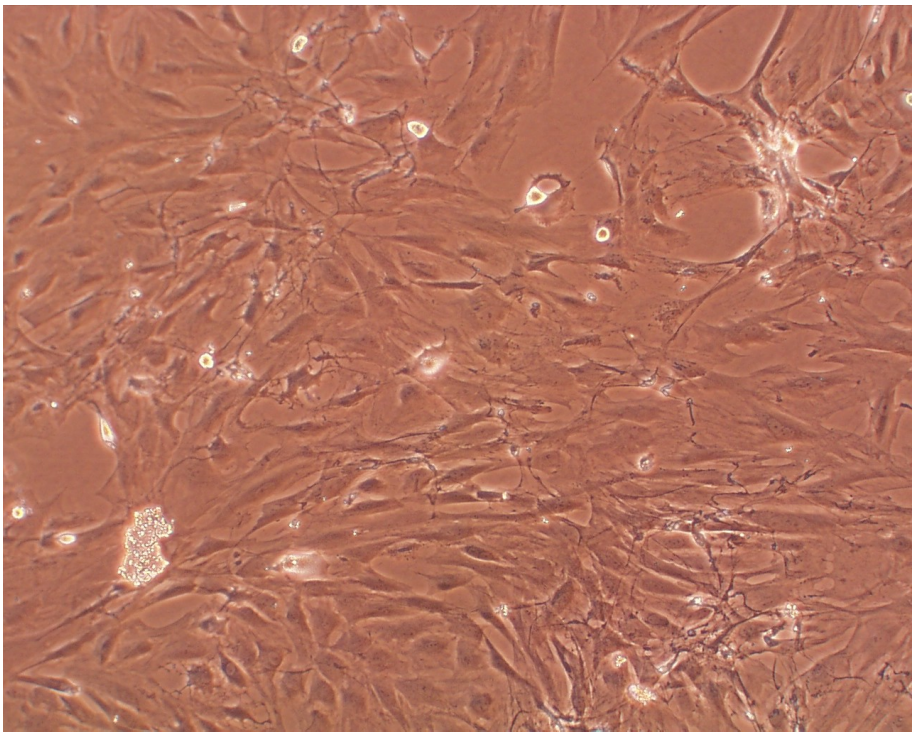

Figure 2.1 Phase contrast photomicrograph of a typical fibroblast culture derived from 19-day fetal rats.

Fibroblasts were isolated from 19-day fetal rats and cultured as described in 2.2.4.1. After at least 8 days in culture a phase contrast photomicrograph was taken using an Olympus DP-10 digital camera in conjunction with an Olympus Model MP-2 inverted phase microscope including an LBT-N filter.

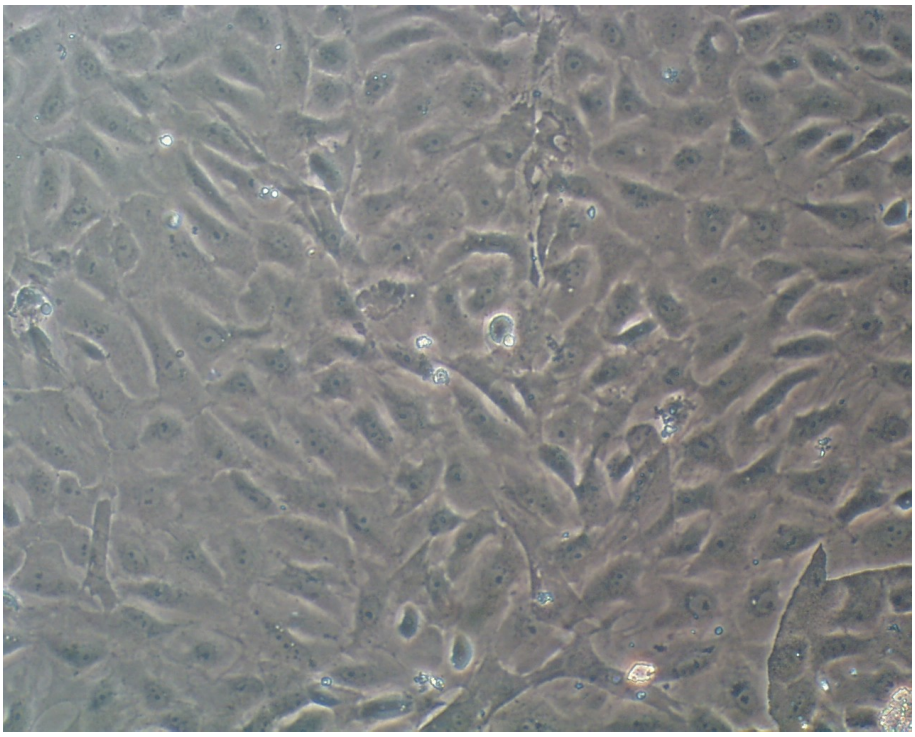

Figure 2.2 Phase contrast photomicrograph of a typical 19-day fetal type II pneumocyte culture.

Type II cells were isolated from 19-day fetal rats and cultured as described in 2.2.4.2. After 4 days in culture a phase contrast photomicrograph was taken using an Olympus DP-10 digital camera in conjunction with an Olympus Model MP-2 inverted phase microscope including an LBT-N filter.

2.2.5 Conditions of cell culture

Cultures were maintained in a water-saturated atmosphere of 5% CO_2 and 95% air at a temperature of 37°C in a Forma Scientific 3250 water-jacketed CO_2 incubator (Forma Scientific, Division of Mallinckrodt Inc., OH, USA). To help maintain a sterile environment, 500 mL of Microshield 5 antiseptic solution (Johnson & Johnson, East London, UK) was added to the water pan, which was changed every three months. Type II cultures were given a media change at 24 hours post-plating. This involved removing media from the plates, washing with 3 mL of BSS and adding 3 mL of MEM^C to each plate. Type II cells were maintained in culture under these conditions for three days prior to changing the media to MEM^+.

2.2.6 Assay of surfactant phospholipid synthesis

2.2.6.1 Effect of heregulin-1β and leptin on phospholipid synthesis in type II cells

Type II cells were prepared as described in 2.2.4. After 72 hours, the plates were removed from the incubator and the media removed. Plates were washed with 3 mL of BSS, and the media replaced with 3 mL of MEM^+. Control plates received 30 µL water, while test plates received 30 µL of the indicated concentrations of either heregulin-1β or leptin. All plates were then incubated for a further 21 hours.

A solution of 7 $\mu C_i.mL^{-1}$ [^{3}H-methyl]-choline chloride in MEM^+ was prepared. The media was removed from each plate into sterilized tubes and 500 µL of the [^{3}H-methyl]-choline chloride solution was added. The tubes were mixed and duplicate aliquots (50 µL) were removed to determine radiolabeled input concentration and the plates returned to the incubator for a further 2, 4 or 6 hour. The plates were then removed from the incubator and placed on ice to prevent further lipid synthesis. The media was removed and the plates washed three times with 3 mL of BSS. The cells were scraped from the plate with 1 mL of BSS followed by another 1 mL and collected

in pre-weighed conical centrifuge tubes, which was kept on ice until all plates had been harvested. The tubes were centrifuged at 1000 *g* at 4°C for two minutes and the supernatant was removed by aspiration and discarded. The centrifuge tubes were again weighed to allow calculation of the weight of cells harvested from each plate. BSS (400 µL) was added to each tube and the cells disrupted using a Braunsonic 1510 sonicator with a microprobe (B. Braun, Melsungen, Germany) at 50 watts for 20 seconds.

Duplicate aliquots (50 µL) of each sonicate were taken and dried in a 50°C oven to determine DNA content. Lipids were extracted from the sonicates using the method of Bligh and Dyer (1959) as follows: A 200 µL aliquot of the sonicate was taken and added to a tube containing 2 mL methanol, 1 mL chloroform, 600 µL of MilliQ H_2O, 50 µL of L-α-phosphatidylcholine 1,2-[1-^{14}C]-dipalmitoyl (approximately 10,000 dpm) as a recovery standard, and 20 µL of 2.5 mg.mL^{-1} dipalmitoyl phosphatidylcholine as a carrier. The mixture was vortexed for 20 seconds and left to stand at room temperature for 10 minutes. This was followed by the addition of 1 mL of chloroform followed by 20 seconds of vortexing. After a further 10 minutes, 1 mL of MilliQ H_2O was added, the tubes vortexed and placed in a 4°C refrigerator for at least 48 hours. Triplicate aliquots (50 µL) of the L-α-phosphatidylcholine 1,2-[1-^{14}C]-dipalmitoyl input solution were also added to polyethylene vials for determination of the input dpm.

During the 48 hours of refrigeration, the chloroform and water/methanol separated into two distinct phases, with the lower phase containing the radioactively labelled lipid. The upper phase was removed by aspiration and duplicate 400 µL aliquots of the lower phase were taken and placed into scintillation vials. The contents of the vial were dried by evaporation for at least three hours and 3 mL of scintillant was added to each vial. The vials were then vortexed and left to stand at room temperature in

the dark for at least 48 hours prior to counting in a Beckman LS3801 scintillation counter (Beckman Instruments, Palo Alto, CA, USA). Tubes were subjected to dual label counting to determine both ^{3}H and ^{14}C. To determine the rate of synthesis of surfactant phospholipids, the following calculation was made:

Surfactant lipid synthesis (dpm.μg DNA^{-1}) =

$$^{3}H \text{ dpm} \quad X \quad \frac{^{14}C \text{ input (dpm)}}{^{14}C \text{ recovered (dpm)}} \quad X \quad \frac{1}{\mu g \text{ DNA}/200\ \mu L}$$

2.2.6.2 Fluorimetric DNA assay

A standard DNA solution was prepared by dissolving highly polymerized calf thymus DNA in MilliQ water to a concentration of 0.1 $mg.mL^{-1}$. Duplicate standards containing 0, 1, 2, 3, 4 and 5 μg of DNA were prepared in separate tubes and dried in a 50°C oven. The amount of DNA was determined using the method of Hinegardner (1971). To each of the standard and test tubes was added 100 μL of a 0.4 $g.mL^{-1}$ 3,5-diaminobenzoic acid solution. They were then placed back in the 50°C oven for a further 45 minutes to allow product development. After this, 1.5 mL of 1.0 M HCl was added to each tube, the contents mixed and aliquots transferred to 96-well plates (Labtech Pty. Ltd., Victoria, Australia), which were then read in a Fluostar Optima plate reader (Walker Sales and Service, Perth, Western Australia) at an excitation wavelength of 405 nm and an emission wavelength of 520 nm. A typical standard curve is shown in Figure 2.3.

2.2.7 Surfactant phospholipid secretion from cultured fetal type II cells

2.2.7.1 Response to heregulin-1β and leptin

The rate of secretion of surfactant phospholipids from type II pneumocytes was determined after exposure of the cells to either heregulin-1β or leptin. After 72 hours of culture, the media was changed to MEM^{+} supplemented with [^{3}H-methyl]-

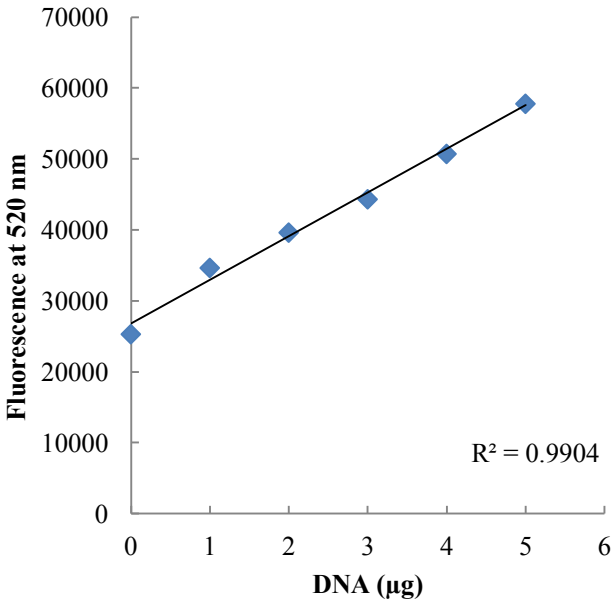

Figure 2.3 A typical standard curve for the fluorimetric assay of DNA.

Standards containing the indicated amounts of highly-polymerised calf thymus DNA were assayed by the method of Hinegardner (1971) as described in 2.2.6.2.

choline chloride (1μCi.mL^{-1}). Following incubation for a further 24 hours, the cultures were washed three times with 3 mL BSS to remove unincorporated choline and any detached cells before adding 1.7 mL of MEM^{+}. The cells were allowed to equilibrate at 37°C in a CO_2 incubator for 1 hour prior to removing two plates to determine the media and cellular content of radiolabelled phospholipids. To the remaining plates (two for each treatment) was added 17 μL of MilliQ water as vehicle (control) or appropriately diluted solutions of heregulin-1β or leptin. The media and cellular content of radiolabelled phospholipids were again determined after 3 hours incubation of the cells with the peptides.

2.2.7.2 Response to (–)-isoproterenol

Type II cells were isolated as described in 2.2.4. The effect of (–)-isoproterenol on the rate of secretion of [^{3}H]-choline-labelled surfactant phospholipids from cultured type II pneumocytes was measured as described by Sen and Cake (1991). After 72 hours growth in culture the medium was changed to MEM^{+} supplemented with [^{3}H-methyl]-choline chloride (1μCi.mL^{-1}). Following further incubation for 24 hours, the cultures were washed with 3 mL BSS three times to remove unincorporated choline and any detached cells prior to adding 1.7 mL of MEM^{+}. The cells were allowed to equilibrate at 37°C in a CO_2 incubator for 1 hour prior to removing two plates for the determination of both the media and cellular content of radiolabelled phospholipids (zero time). During this period of equilibration a 10 mM stock solution of (–)-isoproterenol, which had been prepared in 0.01M HCl and stored for up to 4 weeks at 4°C, was diluted in a solution containing 20 μg.mL^{-1} ascorbic acid to yield working solutions of 10 and 100 μM (–)-isoproterenol. Aliquots (17 μL) of these latter solutions or an equivalent volume of 20 μg.mL^{-1} of ascorbic acid (control) were added to the remaining plates of type II pneumocytes, such that the final

concentration of (–)-isoproterenol was zero, 0.1 or 1 μM. The media and cellular content of radiolabelled phospholipids were again measured after an additional 3 hours of incubation.

2.2.7.3 Response to (–)-isoproterenol after prior exposure to heregulin-1β

Rat type II pneumocytes were isolated from fetal lung tissue and grown in culture for 3 days as described in 2.2.4. The cells were then exposed to 50 $ng.mL^{-1}$ heregulin-1β as well as [^{3}H-methyl]-choline chloride (in order to label surfactant phospholipids). After 24 h exposure the cells were washed three times with 3 mL BSS to remove unincorporated [^{3}H-methyl]-choline chloride and the media replaced with 1.7 mL of MEM^+ and the cells equilibrated for a further 1 hour prior to the addition of 1 μM (–)-isoproterenol or an equivalent volume of ascorbic acid solution (vehicle) as described in 2.2.7.1. Identical experiments were conducted on type II cells derived from either male or female fetal rats.

2.2.7.4 Determination of the level of secretion of surfactant phospholipids from type II cells

The radiolabelled phospholipids in the media and cells were extracted according to the method of Bligh and Dyer (1959). After the cells had been incubated for 3 hours, the plates were placed on ice to prevent further secretion, the medium removed and the cells washed three times with 0.83 mL warm BSS. The media and washings from each plate were combined (4.2 mL total) and vortexed briefly prior to centrifugation for 2 minutes at 1000 *g* (2100 rpm) in a refrigerated GRP centrifuge (Beckman Instruments, Palo Alto, CA, USA) at 4°C to sediment any detached cells. A portion of the supernatant (3.2 mL) was transferred to screw-capped tubes for lipid extraction. The cells from each plate were also transferred into screw-capped tubes using a polyethylene scraper into a mixture of 0.8 mL of MilliQ water and 1.0 mL of

methanol, followed by a further scraping with 1.0 mL of methanol. To each of these tubes was added 50 µL of L-α-phosphatidylcholine 1,2-[1-^{14}C]-dipalmitoyl (approximately 10,000 dpm) as a recovery standard, and 20 µL of 2.5 mg.mL^{-1} dipalmitoyl phosphatidylcholine (DPPC) as a carrier. Triplicate 50 µL aliquots of the L-α-phosphatidylcholine 1, 2-[1-^{14}C]-dipalmitoyl solution was also added directly to polyethylene vials to facilitate the determination of phospholipid recovery. To achieve the required chloroform: methanol: water ratio of 2.0: 2.0: 1.8, 12 mL of chloroform: methanol (1:2) mixture was added to the media samples, and 1 mL of chloroform was added to the cell samples and the mixtures vortexed. After 10 minutes, further additions of 4 mL of chloroform to the media tubes, and 1 mL of chloroform to the cell tubes were made and the mixtures vortexed again. After an additional 10 minutes, 4 mL and 1 mL of MilliQ water was added to the media and cell tubes, respectively. After an additional vortex, the tubes were left to stand overnight at 4°C.

The following day, after careful removal of the upper phase and protein interface (via aspiration), duplicate 3.5 mL aliquots of the chloroform layer of each media extraction tube and a 0.4 mL aliquot of each cell extraction tube were transferred to polyethylene counting vials. The solvent was evaporated from these vials under air at 37°C in a Pierce Reacti-Therm III evaporator over a period of at least 4 hours. Optiphase "HiSafe" II scintillant (3 mL) was added to each vial, which was vortexed and the contents left for at least 48 hours in the dark. Radioactivity was determined in a Beckman LS 6500 liquid scintillation counter (Beckman Instruments Nuclear Division, Fullerton, CA, USA) in which the amounts of both [^{3}H] and [^{14}C] were measured. Finally, the results were expressed in disintegrations per minute and corrected for the recovery of [^{14}C]. Media [^{3}H] counts were also corrected for the fact that only a 3.2 mL aliquot, from a total volume of 4.2 mL, was extracted. The extent of [^{3}H]-phospholipid secretion by the cells was calculated as follows:

% secretion =

$$\frac{\text{dpm in media phospholipids}}{(\text{dpm in media phospholipids})_{+}(\text{dpm in cellular phospholipids})} \times \frac{100}{1}$$

2.2.8 Determination of the β-adrenergic receptor activity in type II cells

Type II pneumocytes derived from mixed sexes or separate male and female 19-day fetal rat lungs as described in 2.2.4, were plated in Primaria 24-well tissue culture plates and maintained in MEM^{C}. The media was changed after 24 hours of incubation and after a further 48 hours the cells were washed twice with 1 mL of BSS, prior to incubation for a further 24 hours with MEM^{+} containing heregulin-1β dissolved in water (final concentration 50 or 100 $ng.mL^{-1}$) or an equivalent volume of water (control). At the end of this incubation, the cells were evaluated for β-adrenergic receptor activity using a technique previously described by Lefkowitz *et al.* (1974). The media was removed and the cells were washed with 170 mM Tris-HCl buffer, pH 7.6 containing 10 μM phenylmethylsulphonyl fluoride, as a protease inhibitor. The cells were then air-dried for a minimum of 2 hours and incubated with 5 nM of the β-adrenergic receptor radioligand, 1-[4,6-propyl-^{3}H]-dihydroalprenolol ([^{3}H]-DHA), dissolved in Tris-HCl. Non-specific binding was determined by incubating the cells with the radioligand in the presence of 10 μM (–)-propranolol. After 2.5 hours incubation, the type II cells were washed three times with 2 mL of Tris-HCl and wiped onto Whatman GF/A glass-fibre filter papers, which were then transferred to polyethylene counting vials for determination of radioactivity. Triplicate 100 μL aliquots of [^{3}H]-DHA dissolved in Tris-HCl with and without 10 μM (–)-propranolol were also counted for quality control purposes.

Optiphase Hisafe-2 scintillant (3 mL) was added to each vial and vortexed prior to measuring radioactivity using a Beckman LS 6500 liquid scintillation counter

(Beckman Coulter Incorporated, Brea, CA, USA). An additional 8 wells, which had not been used for receptor assay, were dried at 50°C and stored for DNA assay as described in 2.2.6.2. The receptor binding, expressed as fmole [^{3}H]-DHA specifically bound per μg of DNA, was determined by subtracting the dpm bound in the presence of (–)-propranolol (non-specific binding) from the dpm bound in its absence (total binding).

2.2.9 Statistical analyses

Results were tabulated and graphed using Microsoft Excel and statistical analyses were carried out using SPSS Statistics 21.0 for Windows. Repeated measures ANOVA (von Ende, 2001) was used to analyze the time course studies and for any differences between 3 or more experimental conditions. If the latter were significant, the ANOVA was followed by Dunnett pairwise comparisons to test for differences from the control group.

2.3 Results

2.3.1 Surfactant phospholipid synthesis

2.3.1.1 Influence of heregulin-1β on surfactant phospholipid synthesis in type II cells

Type II pneumocytes isolated from the lungs of 19-day fetal rats were grown in culture for 72 hours and then incubated for a further 21 hours with 50 ng.mL^{-1} heregulin-1β or vehicle (control) prior to being exposed to 1.0 μCi.mL^{-1} [^{3}H-methyl]-choline chloride. The quantity of radiolabelled surfactant phospholipids synthesised by these cells in the next 2, 4 and 6 hours was enhanced when compared with that which occurred in cells grown in the absence of heregulin-1β (Figure 2.4) This difference between control cultures and NRG1β-treated cells was significant ($p < 0.05$) and, as expected, there was also evidence of an increase in the extent of phospholipid synthesis with time ($p < 0.01$).

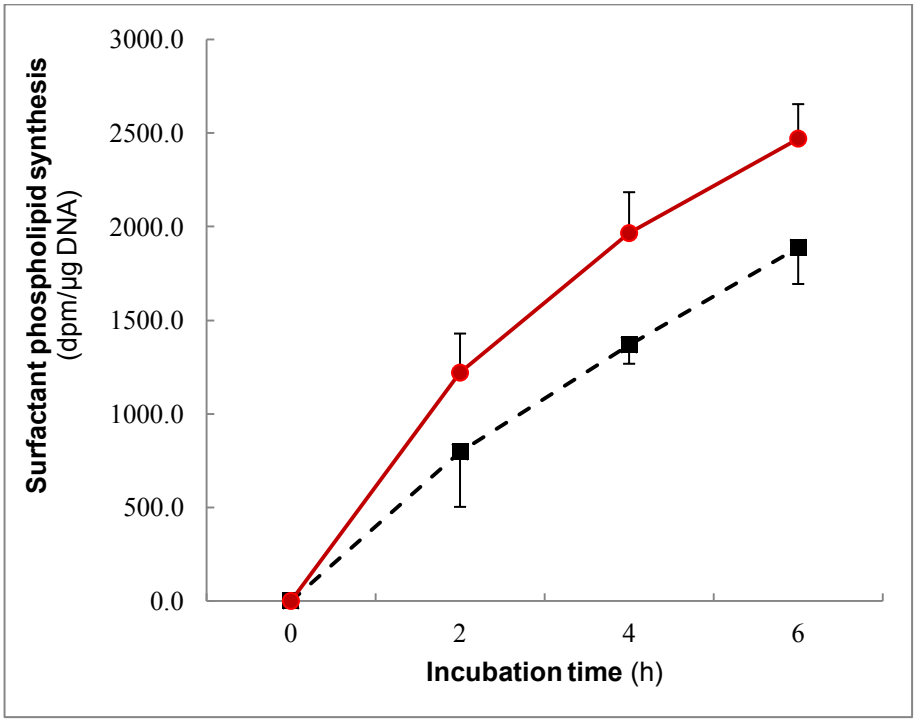

Figure 2.4 **Time course of the effect of heregulin-1β on surfactant phospholipid synthesis**.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in culture for 3 days as described in 2.2.4. The cells were then washed with 3 mL BSS and the media replaced with 3 mL of MEM^{+} containing 50 $ng.mL^{-1}$ heregulin-1β (●) or an equivalent volume of vehicle (control, ■). After 21 h exposure, the cells were then exposed to 1.0 $\mu Ci.mL^{-1}$ [^{3}H-methyl]-choline chloride and re-incubated. The amount of phospholipids synthesised in the next 2, 4 and 6 h was determined as described in 2.2.6. The results represent the mean ± SEM of 8 separate experiments conducted in duplicate.

When type II cells were incubated with 0, 10, 20, 50, 100 or 200 ng.mL^{-1} (i.e. 0 - 26.67 nM) heregulin-1β for 6 hours the quantity of labelled surfactant phospholipids synthesised was higher in those cells exposed to the peptide. However, only for those cells exposed to 20 and 50 ng.mL^{-1} heregulin-1β was this increase significantly different from the control (more than a 3-fold increase; $p < 0.05$). (Figure 2.5).

2.3.1.2 Comparison of the stimulatory effects of heregulin-1β and leptin on the synthesis of surfactant phospholipids in type II cells

Previous studies have shown that leptin stimulates surfactant phospholipid synthesis in rat fetal type II pneumocytes (Torday *et al.*, 2002; Kirwin *et al.*, 2006; Torday and Rehan, 2007). When the stimulatory effects of heregulin-1β and leptin on surfactant phospholipid synthesis were compared, leptin, at both 20 and 50 ng.mL^{-1}, showed a significant increase in surfactant phospholipid synthesis compared to the control ($p < 0.05$ and $p < 0.01$, respectively). In contrast, although heregulin-1β, at a concentration of 50 ng.mL^{-1}, increased the amount of surfactant phospholipids accumulated, this effect was not significantly different from the control (Figure 2.6). This latter finding does not agree with the data shown in Figure 2.5, however, it must be noted that in this experiment with heregulin-1β the labeling time was only 4 h (not 6 hours as in Figure 2.5).

2.3.2 Surfactant phospholipid secretion

2.3.2.1 Influence of heregulin-1β on surfactant phospholipid secretion

When cultured fetal type II cells were incubated in the presence of 50 ng.mL^{-1} heregulin-1β there was a consistent increase in the rate of secretion of surfactant phospholipids from the type II cells as compared to that seen in the control cells (Figure 2.7; $p = 0.05$) and, as expected, there was evidence of an increase in the

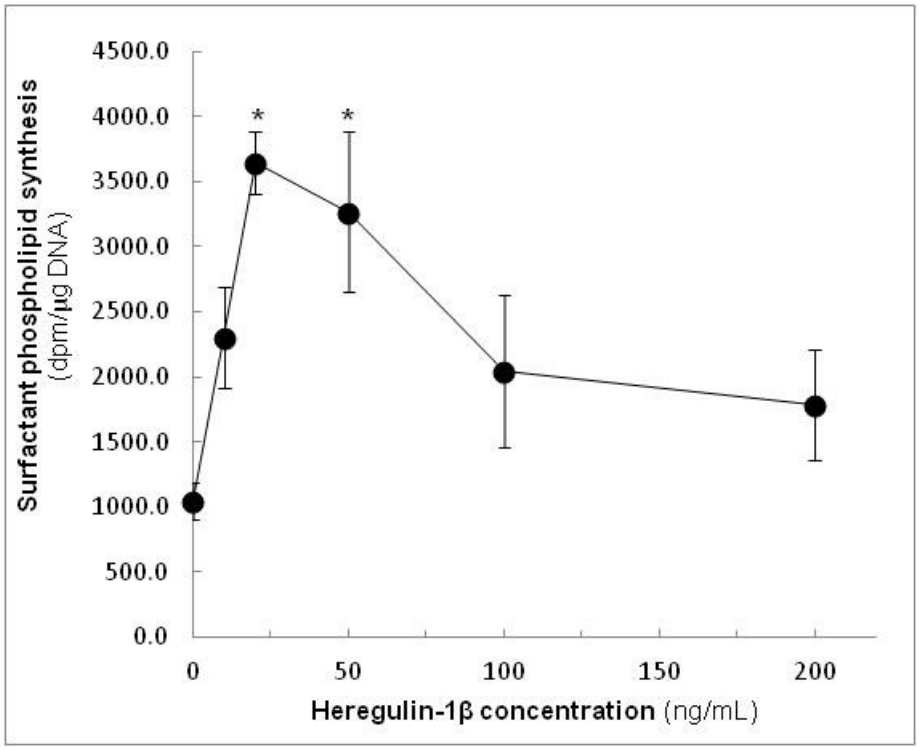

Figure 2.5 Effect of heregulin-1β concentration on surfactant phospholipid synthesis in cultured fetal type II pneumocytes.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in culture for 3 days as described in 2.2.4. The cells were then washed with 3 mL BSS and the media replaced with 3 mL of MEM^{+} containing 10, 20, 50, 100 and 200 $ng.mL^{-1}$ of heregulin-1β (●) or an equivalent volume of vehicle. After 21 h exposure the cells were then exposed to 1.0 $\mu Ci.mL^{-1}$ [^{3}H-methyl]-choline chloride and incubated for a further 6 h. The amount of phospholipids synthesised was determined as described in 2.2.6. The results represent the mean ± SEM of 4 separate experiments conducted in duplicate. Those cells exposed to 20 and 50 $ng.mL^{-1}$ heregulin-1β were significantly different from the control ($p < 0.05$), as indicated by the asterisks.

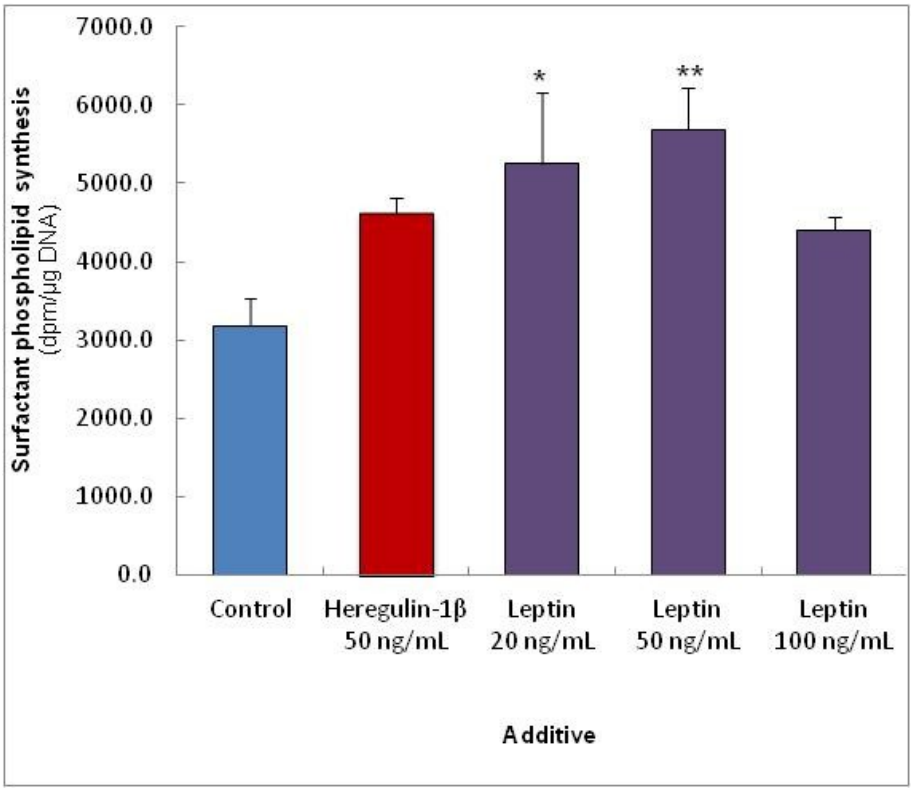

Figure 2.6 Effect of heregulin-1β and leptin on surfactant phospholipid synthesis in cultured fetal type II pneumocytes.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in culture for 3 days as described in 2.2.4. The cells were then washed with 3 mL BSS and the media replaced with 3 mL of MEM^{+} containing 50 ng.mL^{-1} heregulin-1β (■) or 20, 50 and 100 ng.mL^{-1} leptin (■) or an equivalent volume of vehicle (control, ■). After 21 h exposure the cells were then incubated with 1.0 µCi.mL^{-1} [^{3}H-methyl]-choline chloride and incubated for a further 4 h. The amount of phospholipids synthesised was determined as described in 2.2.6. The results represent the mean ± SEM of 4 separate experiments conducted in duplicate. Cells exposed to 20 ($p < 0.05$) and 50 ng.mL^{-1} of leptin ($p < 0.01$) were significantly different from the control, as indicated by the asterisks.

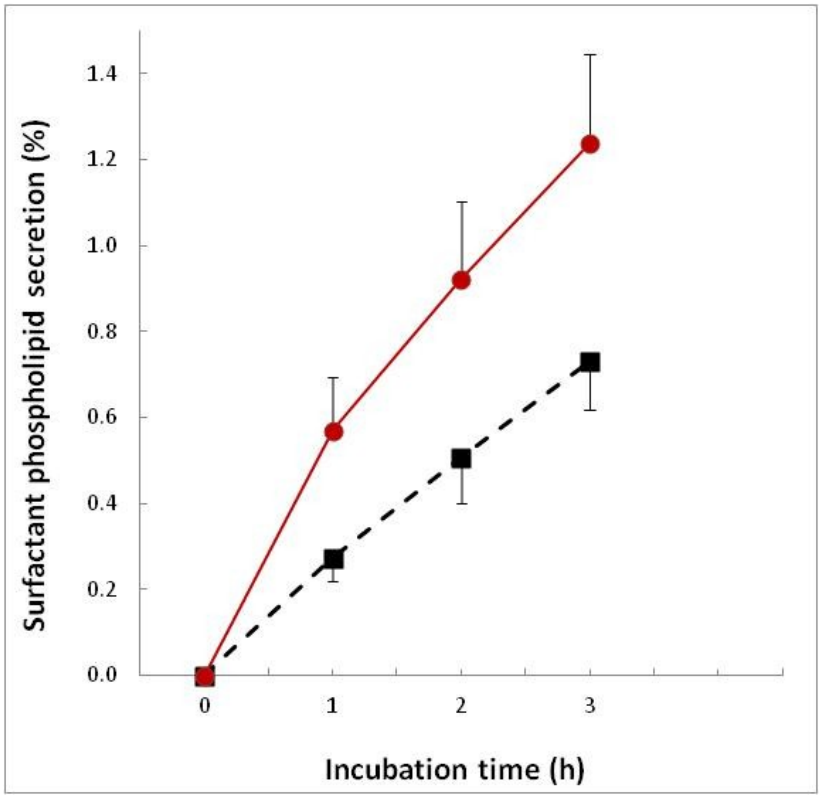

Figure 2.7 Time course of the effect of heregulin-1β on surfactant phospholipid secretion from cultured fetal type II pneumocytes.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in culture for 3 days as described in 2.2.4. The cells were then exposed to 1.0 μCi.mL^{-1} [^{3}H-methyl]-choline chloride in order to label surfactant phospholipids. After 24 h exposure the cells were washed three times with 3 mL BSS to remove unincorporated [^{3}H-methyl]-choline chloride and the media replaced with 1.7 mL of MEM^{+} and the cells equilibrated for a further 1 h prior to the addition of 50 ng.mL^{-1} heregulin-1β (●) or an equivalent volume of vehicle (control, ■). The amount of labelled phospholipid secreted into the media in the next 1, 2 and 3 h was determined as described in 2.2.7.4. The results represent the mean ± SEM of 7 separate experiments conducted in duplicate.

level of phospholipid secretion with time ($p = 0.019$). This effect of enhanced secretion was also evident at 10, 20 and 100 $ng.mL^{-1}$ heregulin-1β, however only at a concentration of 50 $ng.mL^{-1}$ heregulin-1β (2.4-fold; $p < 0.05$) was the rate of secretion significantly different from the control (Figure 2.8).

Whereas leptin had a stimulatory effect on the synthesis of surfactant phospholipids (Figure 2.6), at concentrations of 20-100 $ng.mL^{-1}$ there is no significant effect on the rate of secretion from type II cells. This contrasts with the observation that 50 $ng.mL^{-1}$ heregulin-1β significantly increased both surfactant phospholipid synthesis ($p < 0.05$; Figure 2.5) and secretion ($p < 0.05$; Figure 2.9).

2.3.3 Effect of heregulin-1β on β-adrenergic receptor level in type II pneumocytes

Type II pneumocytes were isolated from 19-day fetal rat lung as described in 2.2.4 and incubated with MEM^{C} for 3 days. The medium was then replaced with MEM^{+} containing 50 or 100 $ng.mL^{-1}$ of heregulin-1β and the cells incubated for a further 24 hours. The control cells had a mean [^{3}H]-DHA binding level of 0.55 $fmol.\mu g^{-1}$ DNA, whereas cells exposed to 50 and 100 $ng.mL^{-1}$ heregulin-1β had binding levels of 0.76 and 0.77 $fmol.\mu g^{-1}$ DNA, respectively, which were both significantly higher than the control level ($p < 0.05$) and corresponded to an approximate 40% increase in the concentration of β-adrenergic receptors (Figure 2.10).

Male and female fetuses from 19-day pregnant rats were separated as described in 2.2.1 and the type II pneumocytes isolated and cultured on 24-well plates as described in 2.2.4.2. The cells were grown for 72 hours in MEM^{C} prior to 24 hours exposure to either 50 $ng.mL^{-1}$ heregulin-1β in 250 μL of MEM^{+} or 250 μL of MEM^{+} alone (control). The cells of both sexes were then analysed for β-adrenergic receptor

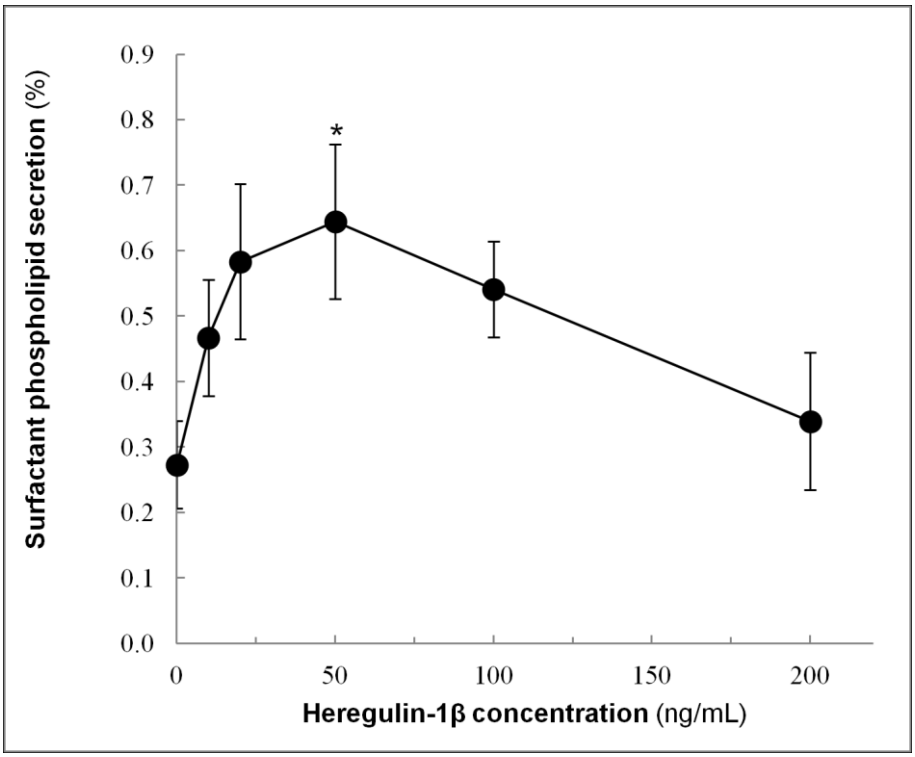

Figure 2.8 Effect of heregulin-1β concentration on surfactant phospholipid secretion from cultured fetal type II pneumocytes.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in culture for 3 days as described in 2.2.4. The cells were then exposed to 1.0 μCi.mL^{-1} [^{3}H-methyl]-choline chloride in order to label surfactant phospholipids. After 24 h exposure the cells were washed three times with 3 mL BSS to remove unincorporated [^{3}H-methyl]-choline chloride and the media replaced with 1.7 mL of MEM^{+} and the cells equilibrated for a further 1 h prior to the addition of 10, 20, 50, 100 and 200 ng.mL^{-1} of heregulin-1β (●) or an equivalent volume of vehicle. After 24 h exposure the cells were exposed to 1.0 μCi.mL^{-1} [^{3}H-methyl]-choline chloride and incubated for a further 3 h. The amount of phospholipids secreted was determined as described in 2.2.7.4. The results represent the mean ± SEM of 6 separate experiments conducted in duplicate. Cells exposed to 50 ng.mL^{-1} heregulin-1β were significantly different from the control ($p < 0.05$), as indicated by the asterisk.

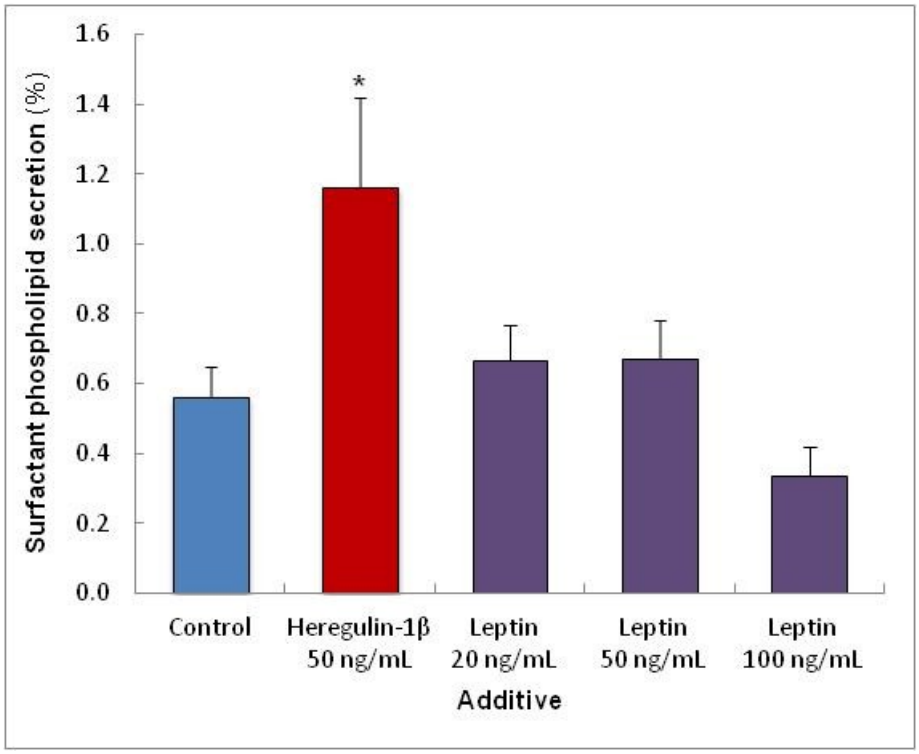

Figure 2.9 Effect of heregulin-1β and leptin on the secretion of surfactant phospholipids from cultured fetal type II pneumocytes.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in culture for 3 days as described in 2.2.4. The cells were then exposed to 1.0 μCi.mL^{-1} [^{3}H-methyl]-choline chloride in order to label surfactant phospholipids. After 24 h exposure the cells were washed three times with 3 mL BSS to remove unincorporated [^{3}H-methyl]-choline chloride and the media replaced with 1.7 mL of MEM^{+} and the cells equilibrated for a further 1 h prior to the addition of 50 ng.mL^{-1} of heregulin-1β (■), 20, 50 or 100 ng.mL^{-1} leptin (■) or an equivalent volume of vehicle (control, ■). The amount of labelled phospholipid secreted into the media in the next 3 h was determined as described in 2.2.7.4. The results represent the mean ± SEM of 5 separate experiments conducted in duplicate. Any result that is significantly different from the control ($p < 0.05$) is indicated with an asterisk.

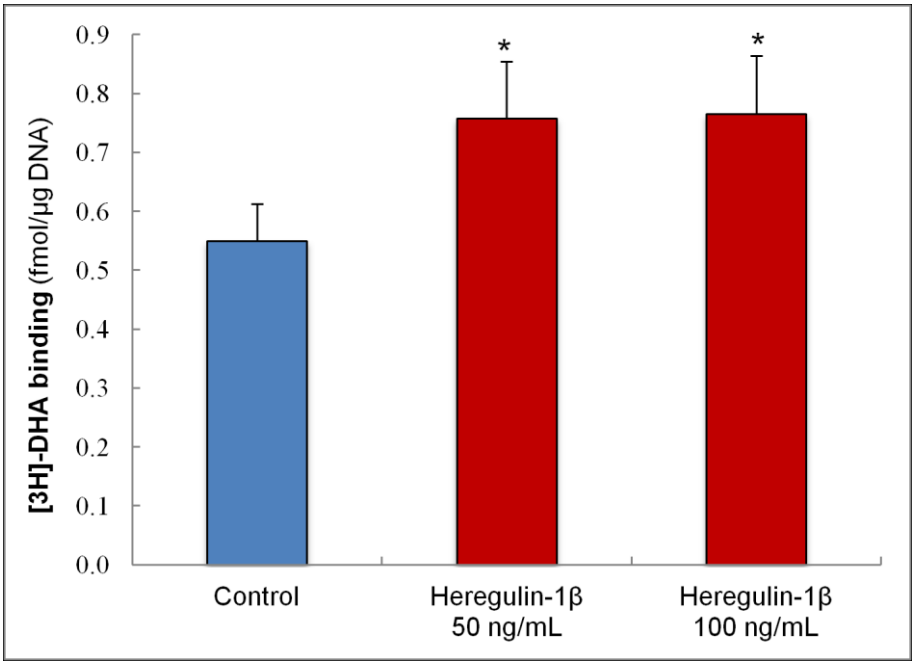

Figure 2.10 Effect of heregulin-1β on β-adrenergic receptor level associated with cultured fetal type II pneumocytes.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in 24–well tissue culture plates for 3 days as described in 2.2.4. Cells were then incubated with 50 or 100 ng.mL^{-1} heregulin-1β (■) or vehicle (control, ■) for 24 h. After being washed thoroughly using BSS, cells were then incubated with [^{3}H]-DHA to allow binding of the radioligand to specific β-adrenergic receptors. The effect of heregulin-1β on the level of β-adrenergic receptors, measured as specific [^{3}H]-DHA binding, was determined as described in 2.2.8. The results represent the mean ± SEM of 10 separate experiments conducted in duplicate. Any result that is significantly different from the control ($p < 0.05$) is indicated with an asterisk. This figure is included with the approval of the author, Liong Ka Hang.

level as described in 2.2.8. For type II cells derived from both male and female fetuses, exposure to heregulin-1β significantly increased the level of β-adrenergic receptor ($p < 0.05$). Moreover, the extent of induction of the β-adrenergic receptor was of similar magnitude in male- and female-derived type II cells (Figure 2.11).

2.3.4 Effect of heregulin-1β on β-agonist stimulation of surfactant phospholipid secretion.

2.3.4.1 Effect of (–)-isoproterenol on surfactant phospholipid secretion.

Fetal rat type II cells were grown in culture for 3 days prior to the surfactant phospholipids being labelled for 24 hours with [^{3}H-methyl]-choline chloride. The amount of labelled phospholipid secreted into the media during a subsequent 3-hour period was 1.4-fold higher in cells exposed to either 0.1 or 1 μM (–)-isoproterenol when compared to the control cells incubated with an equivalent volume of vehicle (ascorbic acid solution), however, this slightly elevated rate of secretion is not significantly different from that seen with control cells (Figure 2.12).

2.3.4.2 Effect of heregulin-1β pre-treatment on (–)-isoproterenol-induced secretion of surfactant phospholipids in type II pneumocytes.

When type II cells were exposed to 50 ng.mL^{-1} heregulin-1β during the period of surfactant phospholipid labelling and subsequently incubated with 1 μM (–)-isoproterenol the response to the β- agonist was significantly greater than that seen in cells exposed only to 1 μM (–)-isoproterenol (Figure 2.13). Whereas (–)-isoproterenol only marginally increases the rate of secretion in control cells (1.2-fold) it induces a 2.5-fold increase ($p < 0.01$) if the cells have been previously exposed to 50 ng.mL^{-1} heregulin-1β. It should be noted that, in those cells exposed to heregulin-1β, the peptide was removed by washing of the cells prior to the addition of the (–)-isoproterenol and the commencement of the 3-hour secretory period. This

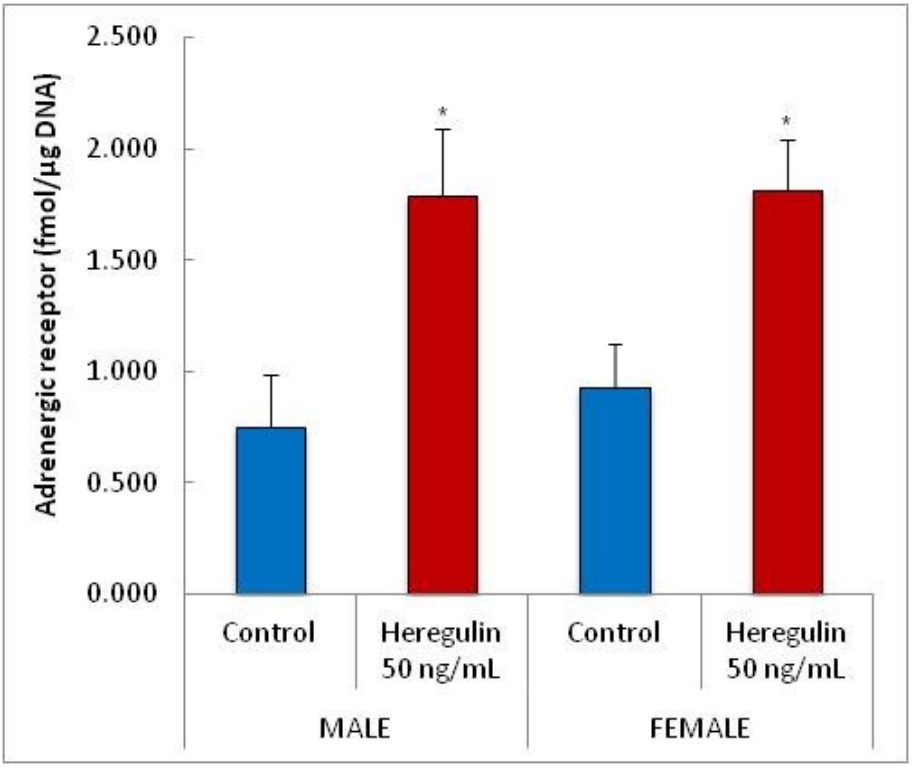

Figure 2.11 Influence of sex on heregulin-1β induction of β-adrenergic receptor level in type II pneumocytes.

Male and female fetuses from 19-day pregnant rats were separated as described in 2.2.1 and the type II pneumocytes isolated and cultured as described in 2.2.4. The cells were grown for 72 hours in MEM^C prior to a 24-hour exposure to either 50 $ng.mL^{-1}$ heregulin-1β in 250 µL of MEM^+ (■) or 250 µL of MEM^+ alone (control, ■). The cells of both sexes were then analysed for β-adrenergic receptor level as described in 2.2.8. The results represent the mean ± SEM of 5 separate experiments conducted in duplicate. Any result that is significantly different from the control ($p < 0.05$) is indicated with an asterisk.

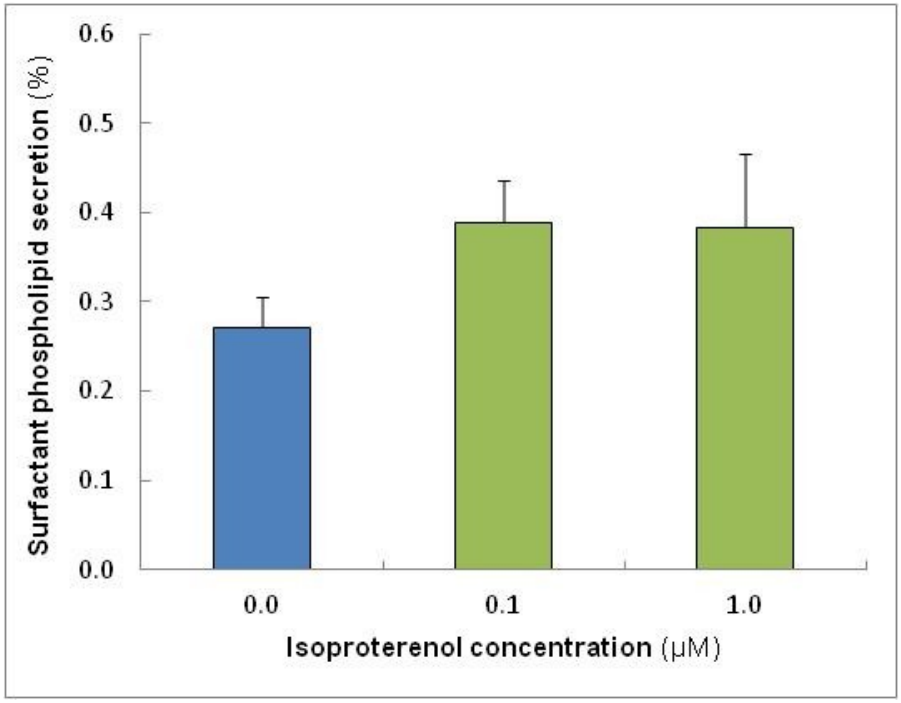

Figure 2.12 Effect of (–)-isoproterenol on surfactant phospholipid secretion.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in culture for 3 days as described in 2.2.4. The cells were then exposed to 1.0 µCi.mL^{-1} [^{3}H-methyl]-choline chloride in order to label surfactant phospholipids. After 24 h exposure the cells were washed three times with 3 mL BSS to remove unincorporated [^{3}H-methyl]-choline chloride and the media replaced with 1.7 mL of MEM^{+} and the cells equilibrated for a further 1 h prior to the addition of 0.1 and 1 µM **(–)**-isoproterenol, respectively (■) or an equivalent volume of ascorbic acid solution (vehicle, ■). The amount of labelled phospholipid secreted into the media after 3 h of incubation was determined as described in 2.2.7.4. The enhanced levels of surfactant secretion in response to 0.1 and 1.0 µM **(–)**-isoproterenol were 1.44-fold and 1.41-fold, respectively. The results represent the mean ± SEM of 4 separate experiments conducted in duplicate.

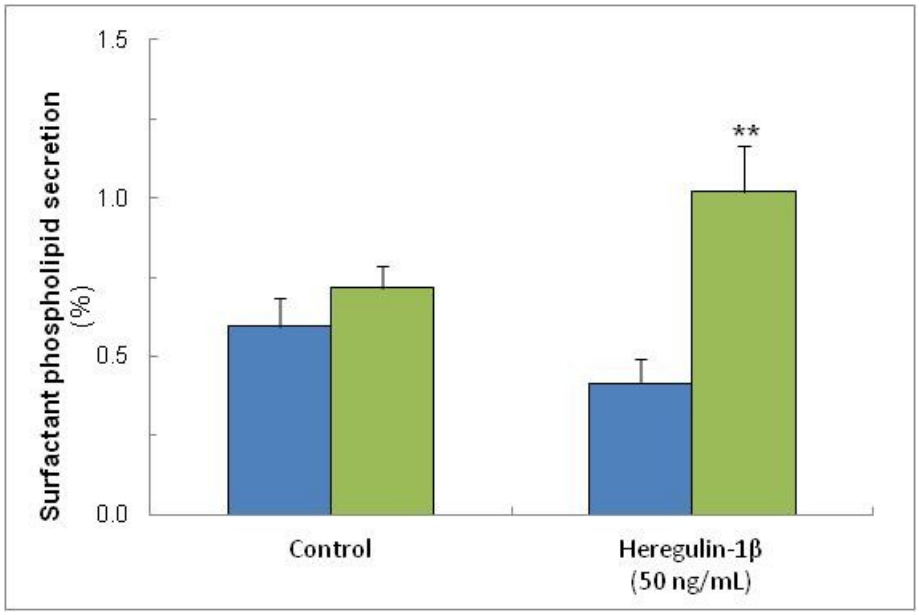

Figure 2.13 Effect of heregulin-1β on (–)-isoproterenol-induced secretion of surfactant phospholipids from type II pneumocyte.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in culture for 3 days as described in 2.2.4. The cells were then exposed to 1.0 μCi.mL^{-1} [^{3}H-methyl]-choline chloride in order to label surfactant phospholipids and 50 ng.mL^{-1} of heregulin-1β. After 24 h exposure the cells were washed three times with 3 mL BSS to remove unincorporated [^{3}H-methyl]-choline chloride, the media replaced with 1.7 mL of MEM^{+} and the cells equilibrated for a further 1 h prior to the addition of 1 μM (–)-isoproterenol (■) or an equivalent volume of ascorbic acid solution (vehicle, ■). The amount of labelled phospholipid secreted into the media after 3 h of incubation was determined as described in 2.2.7.4. The results represent the mean ± SEM of 4 separate experiments conducted in duplicate. Any result that is significantly different from the control ($p < 0.01$) is indicated with asterisks.

stimulatory effect of heregulin-1β on the (–)-isoproterenol-induced secretion of surfactant phospholipids from type II cells was equally evident in cells derived from male and female rat fetuses (Figure 2.14) and similar to that seen in cultures derived from a mixture of male and female fetuses (Figure 2.13).

2.4 Discussion

2.4.1 Surfactant phospholipid synthesis in type II cells

Smith (1978) observed that glucocorticoids had no significant effect on surfactant phospholipid production when applied directly to type II epithelial cells. However, if lung fibroblasts were pre-conditioned in the presence of glucocorticoids, the conditioned media enhanced the rate of phospholipid synthesis in type II cells. They proposed that exposing lung fibroblasts to glucocorticoids generated a differentiation factor, which they called fibroblast pneumocyte factor (FPF). This factor, which was later shown to be a peptide, has a molecular weight of 5-15 kDa (Smith, 1979). Numerous attempts have been made to identify FPF. Torday and Kourembanas (1990) and McDevitt *et al.* (2007) observed that glucocorticoids reduce the expression of TGF-β, an agent which is known to suppress the production of surfactant phospholipids. Torday *et al.* (2002), and subsequently Kirwin *et al.* (2006), provided evidence that leptin, which is expressed by lipofibroblasts during rat lung development, has many of the characteristics of FPF. More recently, Dammann *et al*. (2003) observed that neuregulin-1β is able to mimic the effect of FPF and that the stimulatory effect of FCM can be blocked by pre-treatment with a neuregulin-1β antibody. Despite these numerous attempts to identify FPF its chemical nature is still not definitively known.

In agreement with Smith (1978), Maker (2008) also demonstrated that dexamethasone does not have a stimulatory effect on surfactant phospholipid synthesis when applied directly to type II cells but does so if cultured lung fibroblasts are exposed

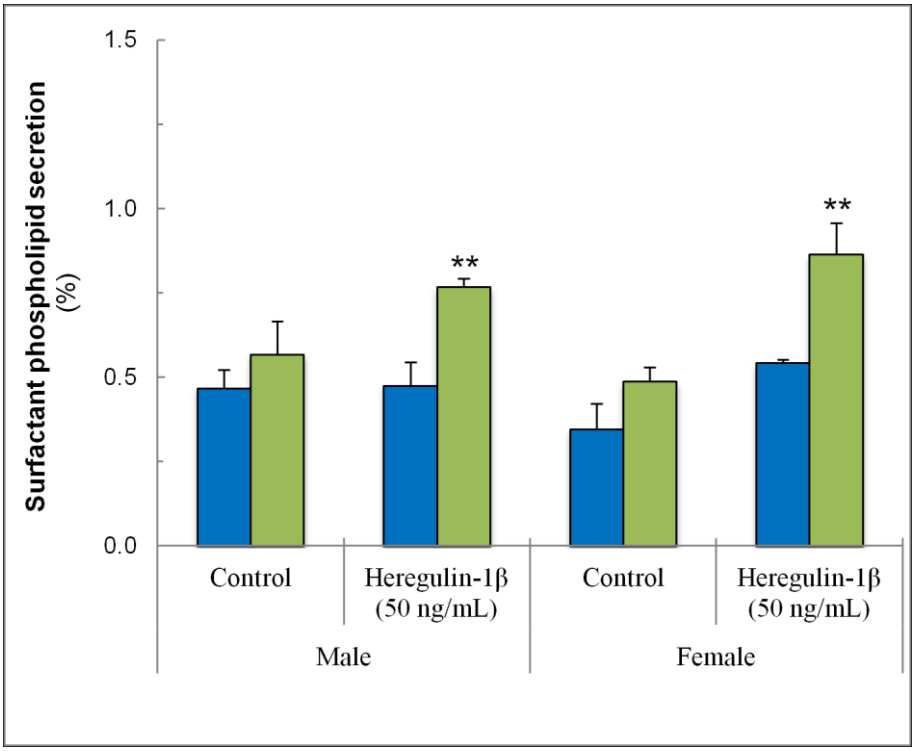

Figure 2.14 Influence of sex of donor animal on the heregulin-1β stimulation of (–)-isoproterenol-induced secretion of surfactant phospholipids.

Fetal rats were separated into male and female groups and then type II cells were isolated from their lungs and grown in culture for 3 days as described in 2.2.4. The cells were then exposed to 1.0 μCi.mL^{-1} [^{3}H-methyl]-choline chloride, in order to label surfactant phospholipids, and 50 ng.mL^{-1} of heregulin-1β. After 24 h exposure the cells were washed three times with 3 mL BSS to remove unincorporated [^{3}H-methyl]-choline chloride and the media replaced with 1.7 mL of MEM^{+} and the cells equilibrated for a further 1 h prior to the addition of 1 μM (–)-isoproterenol (■) or an equivalent volume of ascorbic acid solution (vehicle, ■). The amount of labelled phospholipid secreted into the media after 3 h of incubation was determined as described in 2.2.7.4. The results represent the mean ± SEM of 3 separate experiments conducted in duplicate. Any result that is significantly different from its corresponding control ($p < 0.01$) is indicated with asterisks.

to dexamethasone and the FCM generated under these conditions incubated with the type II cells. Furthermore, using liquid chromatography-mass spectrometry, he showed that FCM generated in the presence of dexamethasone had a higher concentration of neuregulin-1β than that generated in the absence of the steroid, suggesting that neuregulin-1β has, at least, some of the attributes of FPF.

The current study has shown that surfactant phospholipid synthesis increased over time when type II cells were exposed to 50 $ng.mL^{-1}$ heregulin-1β, compared to the corresponding control (Figure 2.4). This linear increase in the incorporation of [^{3}H]-choline into surfactant phospholipids is in agreement with previous findings from this laboratory that radiolabelled phospholipids continue to accumulate within type II cells until approximately 10 hours, when secretion of radiolabelled surfactant phospholipids begins to occur (data not shown). The result is also similar to that observed by Post *et al.* (1986), who showed that there was an elevated rate of incorporation of [^{3}H]-choline into phosphatidylcholine when type II cells were incubated with purified fibroblast pneumocyte factor, although the latter study was conducted over a shorter period of time.

In type II cells exposed to heregulin-1β for six hours over the range 10 to 200 $ng.mL^{-1}$, surfactant phospholipid synthesis was enhanced at all concentrations, but was only significant at 20 and 50 $ng.mL^{-1}$ heregulin-1β compared to the corresponding control ($p < 0.05$; Figure 2.5). It is possible that, at 10 $ng.mL^{-1}$, the ligand concentration was insufficient to saturate the ErbB receptors, whereas at 100 and 200 $ng.mL^{-1}$, the high concentration of the ligand led to down-regulation of the ErbB receptor. Although the mechanism by which this down-regulation occurs in type II cells is unknown, Cao *et al.* (2007) have shown that neuregulin induces both ubiquitination and degradation of the ErbB3 receptor in MCF-7 breast cancer cells. If neuregulin-induced degradation of

the ErbB receptor also occurs in type II cells this could explain the decline in the response to higher concentrations of the ligand. The enhanced rate of phospholipid synthesis in type II cells exposed to neuregulin-1β is in agreement with the observation of Dammann *et al.* (2003) that an antibody to neuregulin-1β was able to completely eliminate the stimulatory effect of FCM on surfactant phospholipid synthesis.

Since Torday *et al.* (2002) have previously shown that leptin was induced in lung fibroblasts exposed to dexamethasone and that leptin enhanced phospholipid synthesis when applied directly to type II cells, a comparison was made between the extent of induction of phospholipid synthesis by leptin and neuregulin-1β. Leptin-treated type II cells showed a significant increase in surfactant phospholipid synthesis when exposed to 20 and 50 $ng.mL^{-1}$ of the ligand for four hours, compared to the control ($p < 0.05$ and $p < 0.01$, respectively) (Figure 2.6). There was also an increase when type II cells were treated with 100 $ng.mL^{-1}$ leptin, but this was not significant ($p > 0.05$). Applying the same argument that was used to explain the optimal concentration for heregulin-1β, it is possible that 20-50 $ng.mL^{-1}$ leptin is the optimal concentration range required to activate leptin receptors. Unlike its effect at six hours, 50 $ng.mL^{-1}$ heregulin-1β did not significantly increase phospholipid synthesis if the exposure time was only four hours, which suggests that the cells require a longer period of exposure to heregulin-1β than to leptin.

2.4.2 Surfactant phospholipid secretion from type II cells

When the rate of secretion of surfactant phospholipids from the type II cells was examined, there was an almost linear increase in the amount of radiolabelled phospholipids secreted under control conditions for up to three hours. In comparison, the rate of surfactant phospholipid secretion was elevated if the cells were exposed to 50 $ng.mL^{-1}$ heregulin-1β (Figure 2.7). If the cells were exposed to heregulin-1β over

the range 10 to 200 ng.mL^{-1} for three hours, secretion was elevated at all concentrations but was only significantly different ($p < 0.05$) from the control rate of secretion at a heregulin-1β concentration of 50 ng.mL^{-1} (Figure 2.8). It is possible at the lower concentrations the ErbB receptors are insufficiently activated to promote the morphological changes required for type II cells to secrete pulmonary surfactant (Crovello *et al.*, 1998). Zhao *et al.*(1998) have shown that the related peptide, recombinant human GGF2 (rhGGF2), which also has its effect via ErbB receptors, enhances embryonic cardiac myocyte proliferation through an elevated rate of thymidine uptake. Similar to the response to heregulin-1β, as reported in this thesis, the effect of rhGGF2 was maximal at a concentration of 30 ng.mL^{-1} and there was a decrease in the response at higher concentrations.

Leptin treatment of type II cells at 20, 50 or 100 ng.mL^{-1} did not bring about any significant change in the level of surfactant phospholipid secretion (Figure 2.9). This finding shows that, whereas leptin stimulates the synthesis of surfactant phospholipids in type II cells (Torday and Rehan, 2002; Torday *et al.*, 2002; Kirwin *et al.*, 2006), it has no effect on the rate at which surfactant is secreted from these cells. This is in contrast to the finding that 50 ng.mL^{-1} heregulin-1β stimulates both synthesis and secretion of the surfactant phospholipids (Figures 2.5 and 2.8).

2.4.3 Influence of sex on heregulin-1β induction of β-AR in type II pneumocytes

Exposure of type II cells to 50 and 100 ng.mL^{-1} heregulin-1β for 24 hours has previously been shown to significantly increase the β-AR level, compared to that in control cells ($p < 0.05$; Figure 2.10). The present study has shown that, irrespective of the gender from which the fetal type II cells were derived, exposure of these cells to heregulin-1β resulted in an enhanced level of β-adrenergic receptors (Figure 2.11).

FCM generated in the presence of dexamethasone has previously been shown to enhance the β-AR activity more if the cells were derived from female instead of male fetuses (Damas, 2010). This suggests that male-derived fibroblasts produce a lower concentration of FPF in response to dexamethasone than those derived from females. Given that neuregulin-1β may be FPF (or at least a component of it), the current study examined the ability of neuregulin-1β to enhance the β-AR activity in cells derived from either male or female fetal lungs. It was observed that the neuregulin-1β-induced activity of β-AR was the same in cells derived from either sex. This observation, together with the previous finding of Damas (Damas, 2010) that there is a sex-linked difference in the induction of β-AR by media conditioned by fibroblasts in the presence of dexamethasone, suggests that the latter difference is an attribute of the lung fibroblasts, a suggestion which is consistent with the previous conclusion of Torday *et al.* (1984).

2.4.4 Effect of heregulin-1β on β-agonist stimulation of surfactant secretion

The rate of secretion of surfactant phospholipid from type II cells after exposure to 1 μM (–)-isoproterenol was slightly elevated, although this increase was not significantly different from the control (Figure 2.12). When type II cells were treated with 50 ng.mL^{-1} heregulin-1β for 24 hours prior to exposure to (–)-isoproterenol, there was a significant increase in the level of surfactant secretion from the cells ($p < 0.01$) (Figure 2.13). This finding is consistent with the observation that heregulin-1β increased β-AR expression in type II cells, which would be expected to enhance the ability of the cells to respond to (–)-isoproterenol. Several investigators have previously shown that (–)-isoproterenol stimulates surfactant phospholipid secretion from lung epithelial cells (Dobbs and Mason (1979); Ormond *et al.*, 2003; Abrahama *et al.*, 2004 and Shi *et al.* (2011) but, to my knowledge, this is the first time that the response to

(–)-isoproterenol has been shown to be modulated by heregulin-1β. This heregulin-1β-induced elevation in the response to (–)-isoproterenol was equally evident if the type II cells were isolated from female or male fetuses (Figure 2.14). This further supports the conclusion that there is no influence of sex on the level of β-AR or β-agonist-induced secretion of surfactant phospholipids from fetal type II pneumocytes.

Chapter 3

Regulation of expression of the neuregulin-1β gene and the β-adrenergic receptor gene in cultured fetal lung cells

3.1 Introduction

3.1.1 Role of neuregulin-β in the response of type II cells to glucocorticoids

Since the pioneering work of Liggins (1969), who demonstrated that glucocorticoids induce precocious maturation of the lung, Smith (1978) has shown that this is an indirect effect resulting from the production of fibroblast pneumocyte factor (FPF) by lung fibroblasts. Subsequently, this release of FPF has also been shown to be elevated if these cells are exposed to EGF (Nielsen, 1989; Sen and Cake, 1991). Work by Dammann *et al.* (2003), together with the findings presented in Chapter 2 of this study, suggest that neuregulin-1β is a strong candidate for being FPF, or at least a major component of FPF. This suggestion is supported by the observation that the concentration of neuregulin-1β in media conditioned by cultured lung fibroblasts in the presence of dexamethasone is increased 2-fold (Maker, 2008). If this is the case, then exposure of lung fibroblasts to either glucocorticoids or EGF would be expected to increase the level of neuregulin-1β mRNA, which is likely to be the consequence of elevated transcription of the neuregulin-1β gene.

Nielsen, Dammann and their co-workers have previously shown that the ErbB receptor family, which includes ErbB1, ErbB2, ErbB3 and ErbB4, and their ligands EGF and neuregulin-1β have a profound influence on the differentiation and maturation of the lung (Dammann *et al.*, 2003). All four ErbB receptors are known to be present in type II cells and have distinct dimerization patterns (Zscheppang *et al.*, 2006; Liu *et al.*, 2007; Zscheppang *et al.*, 2007). In mouse type II cells, ErbB1-ErbB4 and ErbB2-ErbB3 were the most common dimerization pairs and treatment of the cells with neuregulin-1β resulted in enhanced phosphorylation of the tyrosine residues associated with ErbB1 and ErbB2 (Zscheppang *et al.*, 2006; Liu *et al.*, 2007). Subsequent work by this research group has identified ErbB4 as an important receptor in the maturation of

fetal rat type II cells (Zscheppang *et al.*, 2007), and maintenance of surfactant synthesis in adult rat type II cells (Liu *et al.*, 2009).

3.1.2 Role of neuregulin-1β in the glucocorticoid induction of β-adrenergic receptors and the response of type II cells to β-agonists

For a long time it has been known that glucocorticoids stimulate an increase in the pulmonary β-adrenergic receptor level in a diversity of species, including rabbits (Cheng *et al.*, 1980; Giannopoulos and Sommers Smith, 1982), rats (Maniscalco and Shapiro, 1983; McGraw *et al.*, 1995) and mice (Slieker *et al.*, 1995). Glucocorticoids are lipid-soluble hormones which can penetrate the membrane of target lung cells where they interact with their specific intracellular receptors inducing genomic effects via direct DNA binding or transcription factor inactivation (Van der Velden, 1998; Alangari, 2010). The latter author also suggested that some of the effects of glucocorticoids may be the result of non-genomic actions involving interactions with cell membranes or membrane-bound receptors. Mak *et al.* (1995a) have shown that treatment of human lung tissue with dexamethasone increased both the β-adrenergic receptor density and the level of β-AR mRNA, implying that the steroid has its effect through increased transcription of the β-AR gene. It was subsequently reported that there is a glucocorticoid response element (GRE) located at position -379 to -365 in the 5'-flanking region of the rat β-AR gene (Cornett *et al.*, 1998).

Although it is clear from the above that glucocorticoids are able to directly activate transcription of the β-AR gene in type II pneumocytes, it is possible that these steroids may also regulate the level of β-AR via the action of FPF. The latter suggestion comes from the observation that media conditioned by fibroblasts in the presence of dexamethasone induces a higher β-AR density if the fibroblasts are derived from female rather than male fetal rat lungs (Damas, 2010). This implies that this effect of

glucocorticoids might also involve the production of FPF, levels of which are known to be higher if the fibroblasts are derived from female animals (Torday, 1984). Given that neuregulin-1β might be one of the active agents of FPF (see 3.1.1), this study tested the hypothesis that the direct exposure of type II cells to recombinant human neuregulin-1β (heregulin-1β) will either increase transcription of the β-AR gene or act synergistically with dexamethasone to enhance the expression of this gene.

In the current study, qPCR was used to ascertain whether the elevated levels of neuregulin-1β in media conditioned by lung fibroblasts in the presence of dexamethasone was the result of enhanced transcription of the neuregulin-1β gene. In addition, the same technique was employed to determine whether the rate of transcription of the β-AR gene was increased if lung type II cells were exposed to heregulin-1β. This effect was examined at different time intervals for three different concentrations of heregulin-1β in both the presence and absence of 50 nM dexamethasone.

3.2 Materials and methods

3.2.1 Materials

SYBR Green (Invitrogen), which was used in early experiments, was generously provided by Dr. David Berryman, Murdoch University. SYBR Safe DNA gel stain was obtained from Invitrogen, Eugene, OR, USA. Primers and TaqMan probes were obtained from Integrated DNA Technology, Coralville, IA, USA. The SV total RNA isolation system and RNase ONE ribonuclease were purchased from Promega Corporation, Madison, WI, USA. The OneStep RT-PCR kit used for PCR reactions was purchased from Qiagen Sciences, Germantown, MD, USA. Agarose and DTT were purchased from Fisher Biotec, Perth, Western Australia.

3.2.2 Fibroblast cultures for RNA extraction

Fibroblast cells derived from day 19 fetal rat lungs were isolated as described in 2.2.4. After cell growth in MEM^C for at least 8 days, with a media change every other day, the plates were considered confluent with fibroblast cells and free from type II cell contamination. The media was removed from the plates and the cells washed twice with 2 mL of warm BSS. To each plate was then added 3 mL of MEM^+ containing 30 μL of either dexamethasone in propylene glycol (to yield a final dexamethasone concentration of either 20 or 50 nM) or propylene glycol (control), and returned to the incubator. To establish a time course, these additions were made 4, 6, 8 and 24 hours before the cells were harvested.

3.2.3 Type II pneumocyte cultures for RNA extraction

Type II cells derived from day 19 fetal rat lungs were isolated as described in 2.2.4. After cell growth in MEM^C for 3 days, with a media change after one day in culture, the media was removed from the plates and the cells washed twice with 2 mL of warm BSS. To each plate was then added 3 mL of MEM^+ containing 30 μL of either heregulin-1β (to yield a final concentration of 20, 50 or 100 $ng.mL^{-1}$) or water as control. In some experiments the cells were simultaneously exposed to the above concentrations of heregulin-1β with either 50 nM dexamethasone or an equivalent volume of propylene glycol. To establish a time course, these additions were made 2, 4, 6 and 8 hours before cell harvest.

3.2.4 RNA extraction

3.2.4.1 Cell harvesting

Sterile techniques were adhered to at all times during harvesting. After the fibroblast cells had been incubated as indicated, plates were removed from the incubator and the media discarded. The plates were washed 3 times with warm BSS and the cells

transferred to sterile tubes using two additions of 1 mL of BSS and a cell scraper. The combined cell extracts were diluted to 25 mL with BSS and centrifuged at 1000 *g* for 5 minutes. The supernatant was discarded.

3.2.4.2 Extraction procedure

Total RNA from the cell pellet was extracted using the protocol for the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). An Eppendorf 5417C microcentrifuge (Eppendorf AG, Hamburg, Germany) was used for all centrifugation steps. All pipette tips and microcentrifuge tubes used were certified nuclease-free. Following extraction, RNA extracts were stored at -70°C until quantification.

3.2.4.3 RNA quantification

The extracted RNA was quantified using an Agilent 2100 Bioanalyzer with an Agilent RNA 6000 Pico kit (Agilent Technologies Inc., Santa Clara, CA, USA). Prior to use, the extracts were thawed and, when necessary, diluted with RNase-free water to give a mRNA concentration in the range 400-5000 $pg.\mu L^{-1}$. An appropriate aliquot of this solution was added to the RT-PCR such that the final amount of mRNA in each reaction was 5 ng.

3.2.5 Design of RT-PCR primers and probes

To design RT-PCR primers and probes for GAPDH, neuregulin-1β, TGF-β and β-AR genes, an NCBI GenBank search of the cDNA and genomic DNA sequences for these genes was performed on *Rattus norvegicus* chromosome 4 for GAPDH, chromosome 16 for neuregulin-1β, chromosome 1 for TGF-β and chromosome 18 for β-AR. The RT-PCR primers for GAPDH and TGF-β mRNA quantification were designed to include a splice junction between the forward and reverse primers thereby providing the means of distinguishing between the products of mRNA and genomic

DNA based on the size of the product. The reverse primer for neuregulin-1β spanned across a splice junction thus eliminating any contribution by possible genomic DNA contamination. Each of the primer sequences chosen were checked for melting temperature (T_M) using Primer3 software, absence of cross reactivity with other sequences (using BLAST software) and neither primer dimer formation nor interference with reference gene primers in a multiplex reaction, as was evident from agarose electrophoresis of the RT-PCR products. The base sequence of GAPDH, neuregulin-1β, TGF-β and β-AR cDNA, highlighting the primer binding sequences, are shown in Figures 3.1, 3.2, 3.3 and 3.4, respectively. The forward and reverse primer sequences chosen for GAPDH, neuregulin-1β, TGF-β and β-AR are shown in Table 3.1. The primer sequences for Pgk, Rpl13a, ACTB, Ppia and Tfrc, which were obtained commercially as a rat housekeeping gene primer set (Real Time Primers, Elkins Park, PA, USA), are also shown in Table 3.1.

A similar rationale was used to design the TaqMan probes except that their melting temperatures were approximately 10°C higher than either of the forward and reverse primers. The probes for GAPDH, neuregulin-1β, TGF-β and β-AR were designed with Iowa Black® quencher at the 3' end and the FAM (green), Cy5 (red), HEX (yellow) and HEX (yellow) reporter dyes covalently attached at the 5' end, respectively, to allow the different PCR products to be distinguished. The sequence of these probes is shown in Table 3.2.

3.2.6 β-AR primers and test for specificity

Because the β-AR gene has no introns and therefore does not contain splice junctions (Emorine *et al.*, 1987; Kobilka *et al.*, 1987; Chatterjee *et al.*, 1997; Aksoy *et al.*, 2002), the PCR product could theoretically be derived from either mRNA or contaminating genomic DNA. To confirm that the RNA extraction procedure, which

NCBI Reference Sequence: NM_017008

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cDNA - *Rattus norvegicus* (chromosome 4)

ggggctctctgctcctccctgttctagagacagccgcatcttcttgtgcagtgccagcctcgtctcata
gacaagatggtgaaggtcggtgtgaacggatttggccgtatcggacgcctggttaccagggctgccttct
cttgtgacaaagtggacattgttgccatcaacgacccttcattgacctcaactacatggtctacatgtt
ccagtatgactctacccacggcaagttcaacggcacagtcaaggctgagaatgggaagctggtcatcaac
gggaaacccatcaccatcttccaggagcgagatcccgctaacatcaaatggggtgatgctggtgctgagt
atgtcgtggagtctactggcgtcttcaccaccatggagaaggctggggctcacctgaagggtggggccaa
aagggtcatcatctccgcccccttccgctgatgcccccatgtttgtgatgggtgtgaaccacgagaaatat
gacaactccctcaagattgtcagcaatgcatcctgcaccaccaactgcttagcccccctggccaaggtca
tccatgacaactttggcatcgtggaagggctcatgaccacagtccatgccatcactgccactcagaagac
tgtggatggcccctctggaaagctgtggcgtgatggccgtggggcagcccagaacatcatccctgcatcc
actggtgctgccaaggctgtgggcaaggtcatcccagagctgaacgggaagctcactggcatggccttcc
gtgttcctacccccaatgtatccgttgtggatctgacatgccgcctggagaaacctgccaagtatgatga
catcaagaaggtggtgaagcaggcggccgagggcccactaaagggcatcctgggctacactgaggaccag
gttgtctcctgtgacttcaacagcaactcccattcttccacctttgatgctggggctggcattgctctca
atgacaactttgtgaagctcatttcctggtatgacaatgaatatggctacagcaacagggtggtggacct
catggcctacatggcctccaaggagtaagaaaccctggaccacccagcccagcaaggatactgagagcaa
gagagaggccctcagttgctgaggagtccccatcccaactcagcccccaacactgagcatctccctcaca
attccatcccagaccccataacaacaggaggggcctggggagccctcccttctctcgaataccatcaata
aagttcgctgcaccctcaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

Figure 3.1 Base sequence of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

The start and stop codons are highlighted in aqua and purple, respectively, and the sequences in blue correspond to the untranslated regions. The underlined sequences are the primer binding sites for the forward and reverse primers. The sequence highlighted in green corresponds to the binding site for the fam-tagged TaqMan probe for GAPDH cDNA, which was used in all multiplex qPCR analyses. Those bases highlighted in grey represent a splice junction.

NCBI Reference Sequence: NM_031588.1
Neuregulin-1β, cDNA - *Rattus norvegicus* (rat chromosome 16)

gcggccgcagctgccgggagatgcgagcgcagaccggattgtgatcacctttccctcttcgggctgtaag
agagcgagacaagccaccgaagcgaggccactccagagccggcagcggagggacccgggacactagagca
gctccgagccactccagactgagcggacgctccaggtgatcgagtccacgctgcttcctgcaggcgacag
gcgacgcctcccgagcagcccggccactggctcttcccctcctgggacaaacttttctgcaagcccttgg
accaaacttgtcgcgcgtcaccgtcacccaaccgggtccgcgtagagcgctcatcttcggcgagatgtct
gagcgcaaagaaggcagaggcaaggggaagggcaagaagaaggaccggggatcccgcgggaagcccgggc
ccgccgagggcgacccgagcccagcactgcctcccagattgaaagaaatgaagagccaggagtcagctgc
aggctccaagctagtgctccggtgcgaaaccagctccgagtactcctcactcagattcaaatggttcaag
aatgggaacgagctgaaccgcaaaaataaaccagaaaacatcaagatacagaagaagccagggaagtcag
agcttcgaattaacaaagcatccctggctgactctggagagtatatgtgcaaagtgatcagcaagttagg
aaatgacagtgcctctgccaacatcaccattgttgagtcaaacgagttcatcactggcatgccagcctcg
actgagacagcctatgtgtcctcagagtctcccattagaatctcagtttcaacagaaggcgcaaacactt
cttcatccacatcaacatccacgactgggaccagccatctcataaagtgtgcggagaaggagaaaacttt
ctgtgtgaatgggggcgagtgcttcacggtgaaggacctgtcaaacccgtcaagatacttgtgcaagtgc
ccaaatgagtttactggtgatcgttgccaaaactacgtaatggccagcttctacaaagcggaggaactct
accagaagagggtgctgacaattactggcatctgtatcgccctgctggtggtcggcatcatgtgtgtggt
ggcctactgcaaaaccaagaagcagcggcagaagcttcatgatcggcttcggcagagtcttcggtcagaa
cggagcaacctggtgaacatagcgaatgggcctcaccacccaaacccaccgccagagaacgtgcagctgg
tgaatccaatacgtatctaaaaacgtcatctccagtgagcatattgttgagagagaagtggagacttcctt
ttccaccagtcattacacttccacagcccatcactccacgactgtcacccagactcctagtcacagctgg
agtaatgggcacacggagagcgtcatttcagaaagcaactccgtaatcatgatgtcttcggtagagaaca
gcaggcacagcagtcccgccgggggcccacgaggacgtcttcatggcctgggaggccctcgtgataacag
cttcctcaggcatgccagagaaacccctgactcctacagagactctcctcatagcgaaaggtatgtatca
gccatgaccaccccggctcgtatgtcacctgtagatttccacacgccaagctcccctaaatcgccccctt
cggaaatgtctccacccgtgtccagcatgacggtgtccatgccctctgtggcagtcagccccttgtggga
agaagagaggcctctgctgcttgtgacgccaccaaggctacgggagaagaaatatgatcatcacccccag
caactcaactcctttcatcacaaccctgcacatcagagtaccagcctcccccctagcccactgaggatag
tggaggatgaggagtacgagacgacccaggagtatgagtcagttcaagagcccgttaagaaagtcaccaa
tagccggcgggccaaaagaaccaagcccaatggccacattgccaataggttggaaatggacagcaacaca
agttctgtgagcagtaactcagaaagtgagacagaagacgaaagagtaggtgaagacacaccattcctgg
gcatacagaaccccctggcagccagccttgaggtggcccctgccttccgtctggctgagagcaggactaa
cccagcaggccgcttctccacacaggaggaattacaggccaggctgtctagtgtaatcgctaaccaagac
cctattgctgtataaaacctaaataaacacatagattcacctgtaaaactttatttttatataataaagta
tttcaccttaaattaaacaatttatttttatttagcagttctgcaaatagaaaacaggaagaaaaaaaaa
cttttataaattaaatatatgtatgtaaaaatgtgttatgtgccatatgtagcaatttttttacagtatt
tcaaaaacgagaaagatatcaatggtgccttatgttctgttatgtcgagagcaagttttataaagttat
ggtgatttctttttcacagtatttcagcaaaacctcccatatattcagtttctgctggctttttgtgcat
tgcattatgatgttgactggatgtatggtttgcaaggctagcagctcgctcgtgttctctctctctctct
ctctctctctctctctgtctctctctctgtctctctctctctctctctctctctctctctctctctctct
ctctctctctctctgtctctctctctgcttcccgtagctcccaaccagtactgtcttggactggcacatc
catccaaataccttttctactttgtatgaagttttctttgctttcccaatatgaaatgagttctctctact
ctgtcagccaaaggtttgcttcactggactctgagataatagtagacccagcagcatgctactattacgt
atagcaggaaactgcaccaagtaatgtccaataataggaagaaagtaatactgtgatttaaaaaaaaaaa
caaactatattattaatcagaagacagcttgctcttggtaaaaggagctaccattgactctaattttgac
tttttagttattgttcttgacaaagagtaacagcttcaagtacagcctagaaaaaaaaatgggttctggc
ctgctatcaggataaatctatcgacgtagatagattcaactcagtttcactttctgtcttgggggaaatg
atccagccactcatatgacgaccaaccaaccacaggtgcctctgctccctgt

Figure 3.2 Base sequence of the rat neuregulin-1β (NRG-1β) cDNA.

The start and stop codons are highlighted in aqua and purple, respectively, and the sequences in blue correspond to untranslated regions. The underlined sequences are the primer binding sites for the forward and reverse primers. The sequence highlighted in red corresponds to the binding site for the cy5-tagged TaqMan probe for NRG-1β cDNA, which was used in all multiplex qPCR analyses. Those bases highlighted in grey represent a splice junction. Prior to the adenine labelled in red type the sequence gcatcttgggattgaatttatgga, which is found in both of the genes coding for rat GGF and human neuregulin, is missing from the rat neuregulin-1β gene.

NCBI Reference Sequence: NM_021578 + BC076380
Transforming growth factor (TGF-β), cDNA - *Rattus norvegicus* (rat chromosome 1)

ccgcctcccgcaaagacttcaccccaaagctggggcgcacccctgcacgccaccctccccccagcctg
cttcttgagtcccccgcatcccaggaccctctctcctctgggaggccgatctccctcggacctgctggc
aatagcttcctatttaagaacaccccactttgggtcccagagagcgctcatctcgatttttatcccgg
tggcatactgagacactctggtgtcagagcgtcaccgcgactcctgctgctttctccctcaacctcaaa
ttattcaggactatcacctacctttccttgggagaccccaccccacccccacaagccctgcaggggcggg
gcctccgcatcccacctttgcccggggttcgcgctctccgaagttccgtggggcgccgcctcccccatg
ccgccctcggggctgcggctgctgccgcttctgctcccactcccgtggcttctagtgctgacgcccggg
aggccagccgcgggactctccacctgcaagaccatcgacatggagctggtgaaacggaagcgcatcgaa
gccatccgtggccagatcctgtccaaactaaggctcgccagtcccccgagccaggggggaggtaccgccg
ggcccgctgcccgaggcggtgctcgctttgtacaacagcacccgcgaccgggtggcaggcgagagcgct
gacccggagcccgagcccgaggcggactactacgccaaagaagtcacccgcgtgctaatggtggaccgc
aacaacgcaatctatgacaaaaccaaagacatcacacacagtatatatatgttcttcaatacgtcagac
attcgggaagcagtgccagaaccccccattgctgtcccgtgcagagctgcgcctgcagagattcaagtca
actgtggagcaacacgtagaactctaccagaaatatagcaacaattcctggcgttaccttggtaaccgg
ctgctgacccccactgatacgcctgagtggctgtcttttgacgtcactggagttgtccggcagtggctg
aaccaaggagacggaatacagggctttcgcttcagtgctcactgctcttgtgacagcaaagataatgta
ctccacgtggaaatcaatgggatcagtcccaaacgtcgaggtgacctgggcaccatccatgacatgaac
cgacccttcctgctcctcatggccacccccctggaaaggctcaacacctgcacagctccaggcaccgg
agagccctggataccaactactgcttcagctccacagagaagaactgctgtgtacggcagctgtacatt
gactttaggaaggacctgggttggaagtggatccacgagcccaagggctaccatgccaacttctgtctg
gggccctgcccctacatttggagcctggacacacagtacagcaaggtccttgccctctacaaccaacac
aacccgggtgcttccgcatcaccgtgctgcgtgccgcaggctttggagccactgcccatcgtctactac
gtgggtcgcaagcccaaggtggagcagttgtccaacatgatcgtgcgctcctgcaagtgcagctgaggc
cccgccccgccccgccccgcccctcccggcaggcccggccccgccccgccccgcccgcagtgcccgg
ggctgtatttaaggacacctgcacccccccccaagcccatctgggggcccattaaaggtgacagaggaaa
aaaaaaaaaaaaaaaaaaaaaa

Figure 3.3 Base sequence of the rat TGF-β cDNA.

The start and stop codons are highlighted in aqua and purple, respectively, and the sequences in blue correspond to the untranslated regions. The underlined sequences are the primer binding sites for the forward and reverse primers. The sequence highlighted in yellow corresponds to the binding site for the hex-tagged TaqMan probe for TGF-β cDNA, which was used in all multiplex qPCR analyses. Those bases highlighted in grey represent a splice junction.

NCBI Reference Sequence: NM_012492.2
β- Adrenergic receptor, cDNA - *Rattus norvegicus* (rat chromosome 18)

cgcgttcaggctgcagctggcaggcatcgcgagcccggagcacccacgagctcagtgtgcaggacgcgc
ccccagcacagccacctacggtctctgaatgaagcttccaggagtccgcccccggacggctgcgcccca
tcggaggtgcacccgctgagagcgtctgggcactgaaagccggtgcgctcacctgccggccatggagcc
acacgggaatgacagcgacttcttgctggcacccaatggaagccgagcgccaggccacgacatcactca
ggaacgggacgaagcgtgggtggtgggcatggccatcctcatgtcggttatcgtcctggccatcgtgtt
tggcaacgtgctggtcatcacagccattgccaagttcgagcgactacaaaccgtcaccaactacttcat
aacctccttggcgtgtgctgatctagtcatgggcctagcggtggtgccttttggggccagtcatatcct
tatgaaaatgtggaattttggcaatttctggtgcgagttctggacttccattgatgtgttgtgcgtcac
agccagcatcgagaccctgtgcgtgattgcagtggatcgctatgttgctatcacatcgcccttcaagta
ccagagcctgctgaccaagaataaggcccgagtggtcatcctaatggtgtggattgtgtcgggccttac
ctccttcttgcctatccagatgcactggtaccgtgccacccacaagcaagccatcgactgttatgccaa
ggagacttgctgtgacttcttcacgaaccaggcctatgctatcgcttcctctatcgtatctttctacgt
gcccctggtggtcatggtctttgtctattccagggtcttccaggtggccaaaaggcagctgcagaagat
agacaaatccgagggcagatttcatgcccaaaacctcagccaggtggagcaggatgggaggagcgggca
cggactccgaaggtcctccaagttctgcttgaaagagcacaaagccctcaagactttaggcatcatcat
gggcaccttcacctctgctggctgcccttcttcattgtcaatattgtccacgtcatccgggccaacct
catccctaaggaagtttacatcctccttaactggttgggctatgtcaactctgccttcaatcctcttat
ctactgtcggagtccagatttcaggattgccttccaggagcttctgtgccttcgccggtcttcttcgaa
aacctatgggaacggctactctagcaacagcaacggtaggacagactacacaggggagcagagcgcata
tcagctggggcaggagaaagaaaatgaactgctttgtgaggaagcccctggcatggaaggctttgtgaa
ctgtcaaggtactgtgcctagccttagcattgactctcaagggaggaactgtaacacaaacgactcgcc
actgtagtgtaggctttctactctttaagacggcccctccctgacaggacactaaccagactattttac
ttgagtgtaataactttagaataaaattgtatagagattttgcagaaggggcacatccttctgccttttt
ttatttttatttttttaagctgcaagtgagagagagagggagagagagagagagagagagagaaagag
agagagagagactgtatttcagtgcttaatgttttccttgtacagttcagttcctctttgcatggaact
taaaagtttctgtctgaagtgttgttggtctgtctgtttggatggtgtttccatgtatctacctcactg
gtcaagtattaaggataatatatatatatattgctgctggaaattcatatgtaaaggagagagttttct
tcctgtacccttggacttgaaatatcctgtgtcttggacctttctgctgtgaatgtggactctctctca
ctccacttattcgctcaaatggagtgttgtaggcagggatttgagggacaacatcagttgtttttctga
gcaaagtctaaagtttacagtaaataaattgtttgaccatgaaaaaaaaaaaaaaaaaaaaaaa

Figure 3.4 Base sequence of the rat β-adrenergic receptor (β-AR) cDNA.

The start and stop codons are highlighted in aqua and purple, respectively, and the sequences in blue correspond to the untranslated regions. The underlined sequences are the primer binding sites for the forward and reverse primers. The sequence highlighted in yellow corresponds to the binding site for the hex-tagged TaqMan probe for β-AR cDNA, which was used in all multiplex qPCR analyses.

Table 3.1 Forward and reverse primer sequences used in the RT-PCR analyses of GAPDH, neuregulin-1β, TGF-β and β-AR.

Primer	Size (bp)	Forward primer sequence	Reverse primer sequence
GAPDH	207	5'-agacagccgcatcttcttgt-3'	5'-cttgccgtgggtagagtcat-3'
NRG-1β	196	5'-acgactgggaccagccatc-3'	5'-tctggtagagttcctccgctttg-3'
TGF-β	392	5'-gccagatcctgtccaaactaag-3'	5'-gtaacgccaggaattgttgc-3'
β-AR	152	5'-ggagacttgctgtgacttctt-3'	5'-cctcggatttgtctatcttctg-3'
Pgk	253	5'-taaagtcagccatgtgagca-3'	5'-atgaatcccgatgcagtaaa-3'
Rpl13a	242	5'-gtgagggcatcaacatttct-3'	5'-catccgctttttcttgtcat-3'
ACTB	272	5'-cacactgtgcccatctatga-3'	5'-ccgatagtgatgacctgacc-3'
Ppia	295	5'-ctggtggcaagtccatctac-3'	5'-cccgcaagtcaaagaaatta-3'
Tfrc	242	5'-catctccatctgaccctcac-3'	5'-gtctttggcttctggtgtca-3'

Table 3.2 Probe sequences used in the qPCR analyses of GAPDH, neuregulin-1β, TGF-β and β-AR.

Probe	Dye	TaqMan probe sequence
GAPDH	FAM	5'(FAM)-ccgtgtgaacggatttggccgtatc-(IABkFQ) 3'
NRG-1β	Cy5	5'(Cy5)-tggcaacgatcaccagtaaactcatttgg-(IAbRQSp) 3'
TGF-β	HEX	5'(HEX)-tcgctttgtacaacagcacccgc-(IABkFQ) 3'
β-AR	HEX	5'(HEX)-cgtatctttctacgtgcccctggt-(IABkFQ) 3'

includes the use of DNase, is sufficient to completely remove any contaminating genomic DNA, agarose gels were run of the RT-PCR products generated from three independent cellular RNA extracts previously incubated with or without 1.5-2.0 U RNase. If the RT-PCR products are derived only from mRNA, and not contaminating genomic DNA, this RNase treatment should eliminate all bands. These experiments were carried out as detailed in Table 3.3.

3.2.7 Quantitative reverse transcriptase polymerase chain reaction

To measure relative gene expression, QIAGEN OneStep RT-PCR was undertaken using a QIAGEN Rotor-Gene Q6000 (QIAGEN, Hilden, Germany). For each RNA extract a PCR reaction was set-up in a UV-sterilised, RNase-free PCR tube (Axygen - Fisher Biotec, Perth, Western Australia), containing forward and reverse primers as well as the TaqMan probes for the reference gene and the genes of interest. The total volume for each reaction was 25 μL as shown for GAPDH, NRG-1β and TGF-β in Table 3.4 and for GAPDH and β-AR in Table 3.5.

In order to optimize the RT-PCR conditions, a test was conducted by varying the annealing temperature between 55-59^{o}C and it was established that 57^{o}C provided the best specificity and the highest yield of product. Thus the reaction mixtures were incubated at 50°C for 30 minutes followed by denaturation at 95^{o}C for 15 minutes. This was followed by 40 cycles using the following parameters; 94°C for 30 sec; 57°C for 30 sec and 72°C for 60 sec. The reactions were then held at 72°C for 10 minutes. The RT-PCR products were then either subjected to agarose gel electrophoresis or the expression of the test genes relative to GAPDH was ascertained using the method of Pfaffl (2001).

Table 3.3 Pre-treatment of cellular RNA extracts with RNase.

RNA extracts were subjected to the following incubation and 3.8 μL of each of the incubations (± RNase), equivalent to 5 ng of RNA, was subjected to multiplex RT-PCR.

	-RNase	**+RNase**
mRNA template (27.78 ng)	10 μL	10 μL
10x TNE buffer	2 μL	2 μL
RNase-free water	8 μL	4 μL
RNase solution (1.5-2.0 U)	-	4 μL
Incubate at 37°C for 30 minutes		
105 mM DTT in RNase-free water	1 μL	1 μL
Incubate at 70°C for 20 minutes		

Table 3.4 Components for a OneStep multiplex qPCR reaction for NRG-1β.

Component	Volume (μL)	Final concentration
5xQIAGEN OneStep RT-PCR	5.0	
dNTP	1.0	400 μM of each dNTP
QIAGEN OneStep RT-PCR	1.0	
GAPDH forward/reverse primers	0.5	0.2 μM
NRG-1β forward/reverse primers	0.5	0.2 μM
TGF-β forward/reverse primers	0.5	0.2 μM
GAPDH probe	0.5	50 nM
NRG-1β probe	0.5	50 nM
TGF-β probe	0.5	50 nM
RNA extract from fibroblasts	Aliquot equivalent to 5 ng	200 ng/mL
RNase-free water	Water to final volume = 25μL	

Table 3.5 Components for a OneStep multiplex qPCR reaction for β-AR.

Component	Volume (μL)	Final concentration
5xQIAGEN OneStep RT-PCR	5.0	
dNTP	1.0	400 μM of each dNTP
QIAGEN OneStep RT-PCR	1.0	
GAPDH forward/reverse primers	0.25	0.1 μM
β-AR forward/reverse primers	1.0	0.4 μM
GAPDH probe	0.5	50 nM
β-AR probe	0.5	50 nM
RNA extract from type II cells	Aliquot equivalent to 5 ng	200 ng/mL
RNase-free water	Water to final volume = 25μL	

3.2.8 Agarose gel electrophoresis

Agarose gels were prepared by dissolving 1.5 g molecular biology grade agarose in 100 mL of 40 mM Tris-HCl buffer, pH 8.0, containing 20 mM acetic acid, 10 mM EDTA and the mixture heated in a microwave until the agarose was completely dissolved. After cooling 1.5 μL of SYBR Safe solution (Invitrogen) was added, the gels were poured and allowed to set in a 4^{o}C cold room for approximately 30 minutes. An aliquot of each RT-PCR incubation mixture (Section 3.2.7) was mixed with loading buffer containing bromophenol blue, xylene cyanol FF in 30% glycerol. Aliquots (5 μL) of each sample and 5 μL of a 100-3000 bp DNA ladder (Axygen - Fisher Biotec, Perth, Western Australia) were loaded onto an agarose gel. Electrophoresis was performed in a BioRad slab gel electrophoresis unit (a wide, mini-sub cell GT) using a BioRad power Pac 300 set at 90 V until the dye was almost at the end of the gel. A photograph of the gel was taken under blue light using a Vilber Lourmat Bio-Vision+1000 camera (Fisher Biotec, Perth, Western Australia).

3.2.9 Statistical analyses

Results were tabulated and graphed using Microsoft Excel and statistical analyses were carried out using SPSS Statistics 21.0 for Windows. Repeated measures ANOVA was used to analyze for the differences between 3 or more experimental conditions. If significant, the ANOVA was followed by Dunnett pairwise comparisons to test for differences from both the control and dexamethasone groups.

3.3 Results

3.3.1 Effect of dexamethasone on neuregulin-1β gene expression

3.3.1.1 Gel of singleplex GAPDH, neuregulin-1β and TGF-β RT-PCR products

The products from singleplex RT-PCR reactions, carried out using RNA extracts from cultured rat lung fibroblasts, grown under control conditions, and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), neuregulin-1β (NRG-1β) or

transforming growth factor-β (TGF-β) primers, were electrophoresed on an agarose gel, as described in 3.2.8. For each of these primers a single band was observed of the size expected for the corresponding pair of primers (i.e. GAPDH 207 bp; neuregulin-1β 196 bp; and TGF-β 392 bp) (Figure 3.5). The GAPDH and NRG-1β PCR products, which are of similar size, migrate to a similar position (i.e. adjacent to the 200 bp band of the DNA ladder), whereas the TGF-β PCR product expectedly migrates adjacent to the 400 bp band of the DNA ladder.

3.3.1.2 Gel of the products derived from both a multiplex and singleplex neuregulin-1β RT-PCR

The products from RT-PCR, carried out using either multiplex PCR with primers for GAPDH, neuregulin-1β and TGF-β or a singleplex PCR using only primers for neuregulin-1β, were electrophoresed on an agarose gel. As the primers for GAPDH and neuregulin-1β yield products of similar size, which are distinct from those generated using TGF-β primers, only two bands were observed under multiplex conditions. The singleplex product generated using only neuregulin-1β primers yielded a single band, which corresponded to the smaller of the two multiplex bands. It is possible that, under multiplex conditions, separate GAPDH and NRG-1β PCR products are generated but because of their similar size they are not separated on the agarose gel (Figure 3.6). Such a conclusion was verified when the multiplex PCR products from similar fibroblast RNA extracts were run on an Agilent 2100 Bioanalyser producing three separate bands corresponding to 199, 214 and 392 bp, which are very close to the expected size of NRG-1β, GAPDH and TGF-β products, respectively (Figure 3.7).

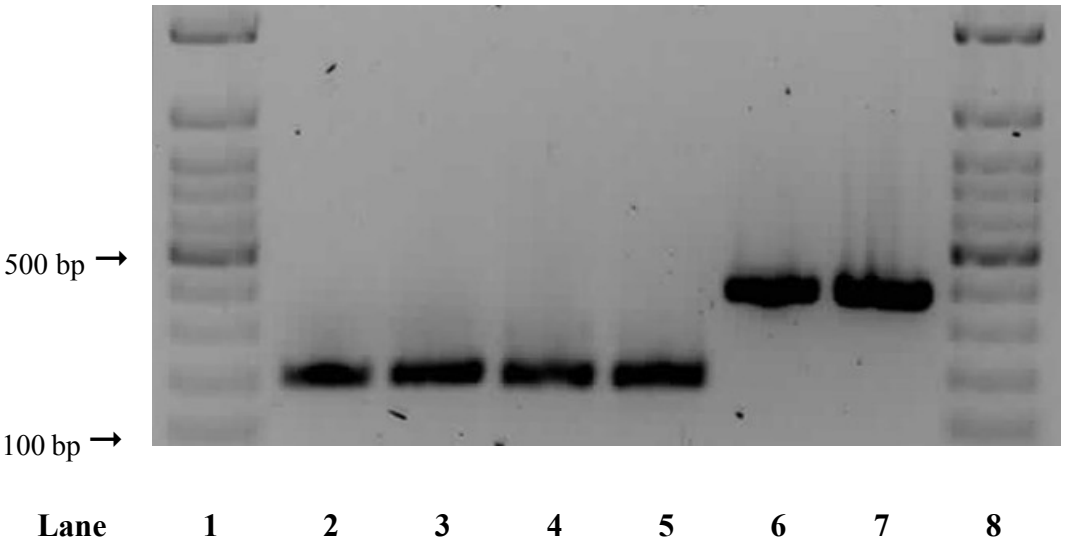

Figure 3.5 Agarose gel of the singleplex RT-PCR products using GAPDH, neuregulin-1β and TGF-β primers.

RNA from cultured rat lung fibroblasts was extracted and quantified as described in 3.2.4.2 and 3.2.4.3, respectively. Singleplex RT-PCR was carried out using primers for GAPDH, neuregulin-1β and TGF-β. An agarose gel was prepared and the RT-PCR products run as described in 3.2.8. The GAPDH and NRG-1β primers yielded single bands of approximately 200 bp whereas the TGF-β primers yielded a single band of approximately 400 bp. *Lanes 1 and 8 - 100-3000 bp DNA ladder; lanes 2 and 3 - GAPDH; lanes 4 and 5 - neuregulin-1β; lanes 6 and 7 - TGF-β.*

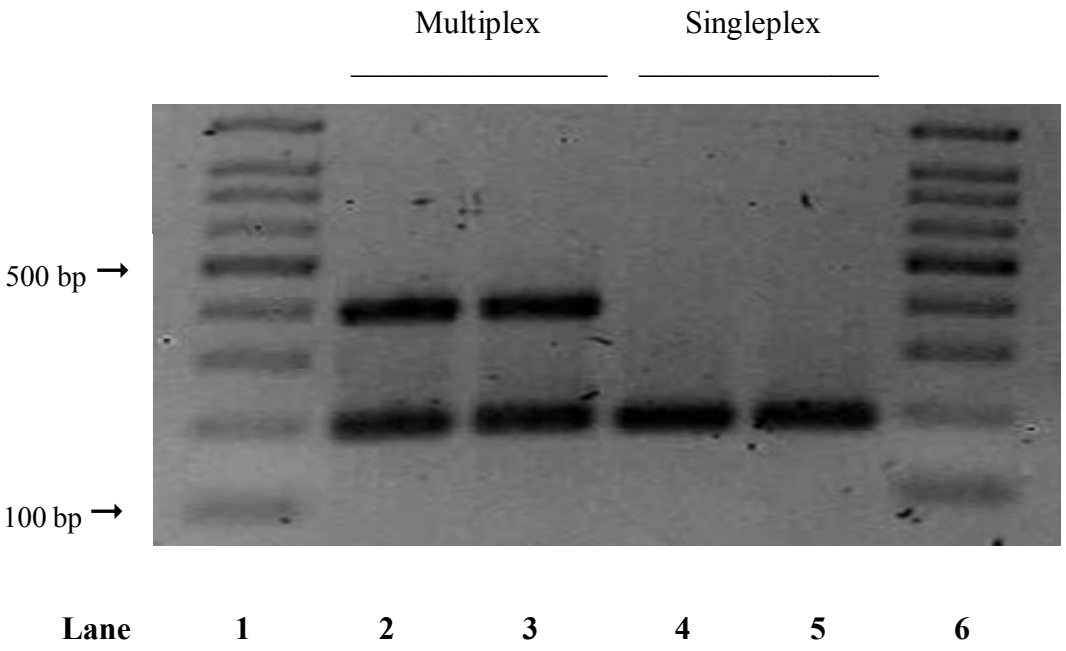

Figure 3.6 Agarose gel of multiplex (GAPDH/neuregulin-1β/TGF-β) and singleplex neuregulin-1β RT-PCR products.

RNA from cultured rat lung fibroblasts was extracted and quantified as described in 3.2.4.2 and 3.2.4.3, respectively. Both multiplex RT-PCR, using primers for GAPDH, neuregulin-1β and TGF-β, and singleplex RT-PCR, using only neuregulin-1β primers, were carried out in duplicate. An agarose gel was prepared and the RT-PCR products run as described in 3.2.8. *Lanes 1 and 6 – 100-3000 bp DNA ladder; lanes 2 and 3 – multiplex GAPDH/neuregulin-1β/TGF-β; lanes 4 and 5 - singleplex neuregulin-1β.*

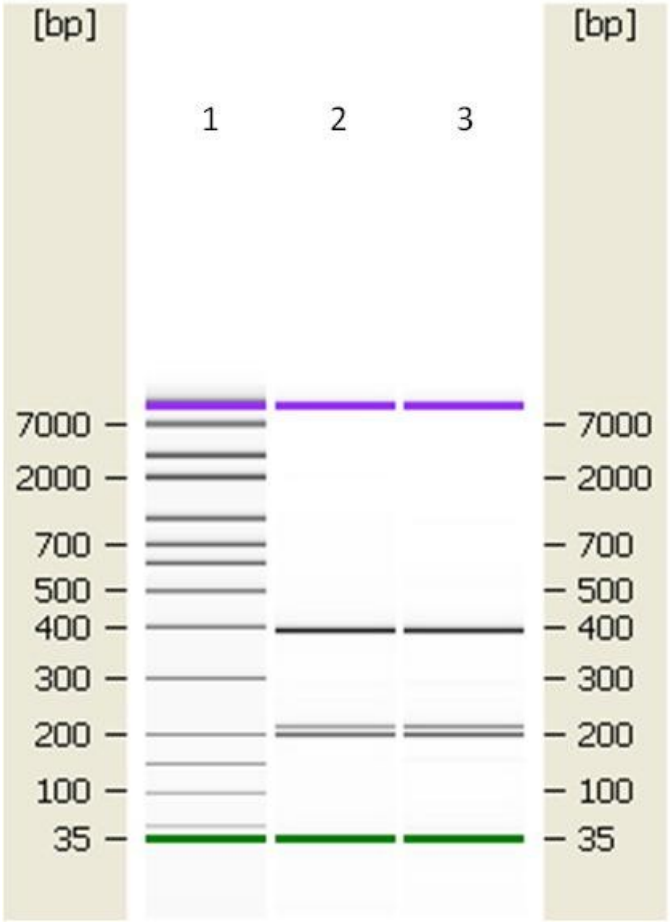

Figure 3.7 Separation of multiplex GAPDH, neuregulin-1β and TGF-β PCR products on an Agilent 2100 Bioanalyzer.

RNA extracts from cultured rat lung fibroblasts were prepared as described in the legend to Figure 3.6. Multiplex RT-PCR was carried out using primers for GAPDH, neuregulin-1β, and TGF-β and the products separated using an Agilent 2100 Bioanalyzer. *Lane 1 - 50-7000 bp DNA ladder; lanes 2 and 3 - GAPDH/neuregulin-1β/ TGF-β multiplex products from two separate RNA extracts.*

3.3.1.3 Standard curves for qPCR of fibroblast mRNA extracts

Multiplex qPCR was carried out using primers and probes for GAPDH (FAM), neuregulin-1β (Cy5) and TGF-β (HEX) with cultured rat lung fibroblast RNA extracts either undiluted or diluted 10-, 100-, 1000- or 10,000-fold. As the primers for GAPDH and neuregulin-1β yield products of similar size, which are distinct from that generated using TGF-β primers, only two bands were observed on the gel. Because the qPCR was run for 40 cycles, a decrease in the intensity of the bands was only evident at the two lowest concentrations of template (Figure 3.8). Screen images using the QIAGEN Rotor-Gene Q 6000 software were captured to show the result for each of the probes - GAPDH (FAM), neuregulin-1β (Cy5) and TGF-β (HEX).

The cycle threshold values (Ct) for each of GAPDH (FAM [green] channel), neuregulin-1β (Cy5 [red] channel) and TGF-β (HEX [yellow] channel) increased as the template concentration decreased. For the template concentration range of 0.022-219.2 $pg.\mu L^{-1}$ the Ct values declined from 31.38-19.45 for GAPDH, from 36.5-23.6 for NRG-1β and from 37.7-25.3 for TGF-β (Figure 3.9). Standard curves for GAPDH, NRG-1β and TGF-β were established by plotting the corresponding Ct values against the template concentration. Each curve revealed a linear relationship between these two parameters ($R^2 \geq 0.99$; see Figures 3.10, 3.11 and 3.12). The efficiency (E) values were estimated to be 113%, 84% and 109% for GAPDG, NRG-1β and TGF-β, respectively.

3.3.1.4 Effect of dexamethasone on neuregulin-1β and TGF-β gene expression in cultured rat lung fibroblasts

When cultured rat lung fibroblasts were exposed to dexamethasone (20 or 50 nM) or an equivalent volume of propylene glycol (vehicle), the steroid was shown to have no effect on the expression of either the neuregulin-1β or TGF-β gene,

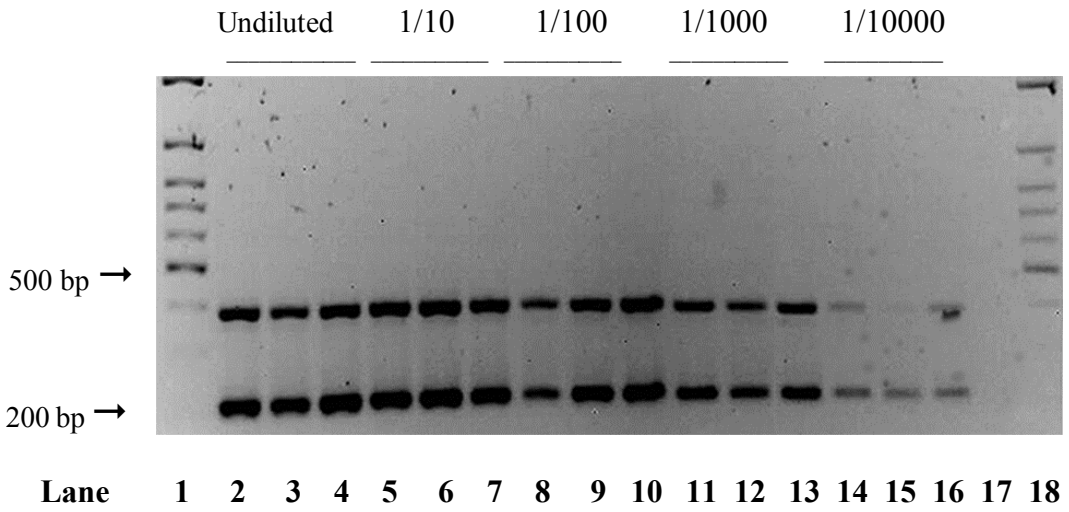

Figure 3.8 **Gel of the multiplex (GAPDH/NRG-1β/TGF-β) PCR products generated with different concentrations of the fibroblast mRNA extract.**

RNA from cultured rat lung fibroblasts was extracted and quantified as described in 3.2.4.2 and 3.2.4.3, respectively. Multiplex RT-PCR was carried out in triplicate using GAPDH, neuregulin-1β and TGF-β primers at five different template concentrations. An agarose gel was prepared and the RT-PCR products run as described in 3.2.8. *Lanes 1 and 18 - 100-3000 bp DNA ladder; lanes 2, 3 and 4 – undiluted template; lanes 5, 6 and 7 - 1/10 diluted template; lanes 8, 9 and 10 - 1/100 diluted template; lanes 11, 12 and 13 - 1/1000 diluted template; lanes 14, 15 and 16 - 1/10000 diluted template; lane 17 - water.*

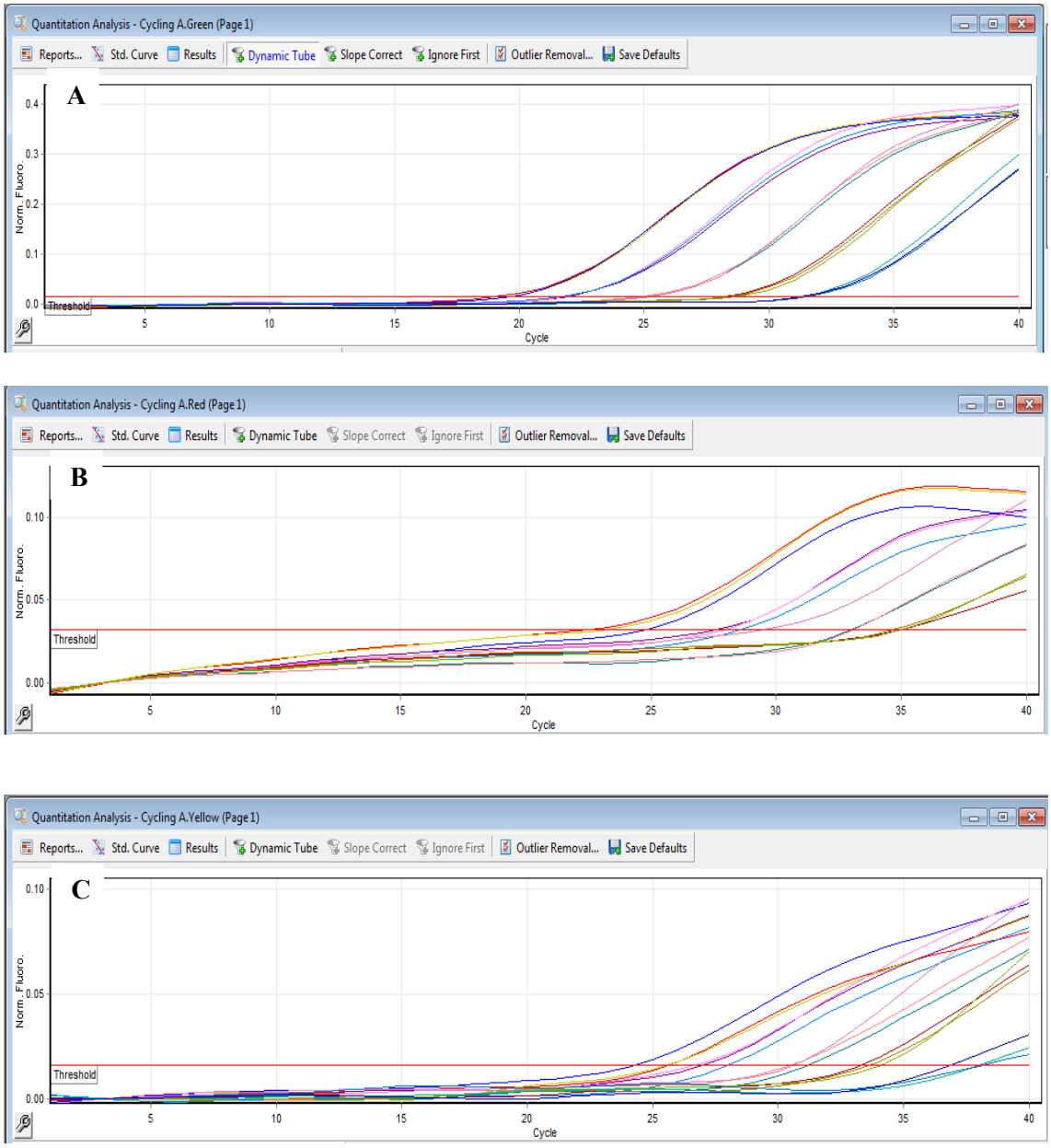

Figure 3.9 Amplification profiles of the GAPDH, NRG-1β and TGF-β standard curves.

The fibroblast mRNA extraction and analytical procedures are as described in Figure 3.8. Screen images of the cDNA amplification profiles at five different template concentrations were captured using the (A) FAM (green), (B) Cy5 (red) and (C) HEX (yellow) channels to detect binding of the specific probes for GAPDH, NRG-1β and TGF-β, respectively.

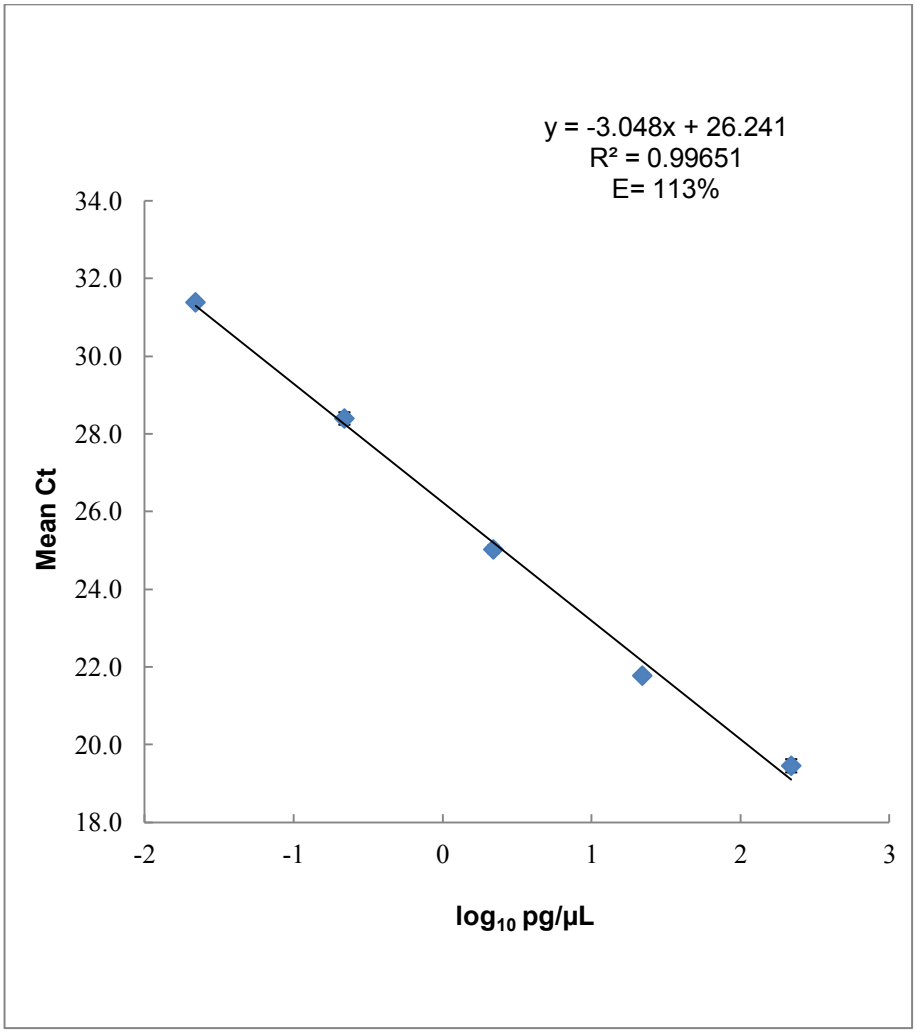

Figure 3.10 **Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) standard curve from the multiplex qPCR products of fibroblast mRNA extracts.**

The fibroblast mRNA extraction and analytical procedures are as described in Figure 3.8. The extract was subjected to multiplex qPCR at various dilutions and then analyzed using a FAM-labeled GAPDH probe to ascertain the Ct values at each concentration of the extract. Each data point represents the mean ± SEM of triplicate determinations.

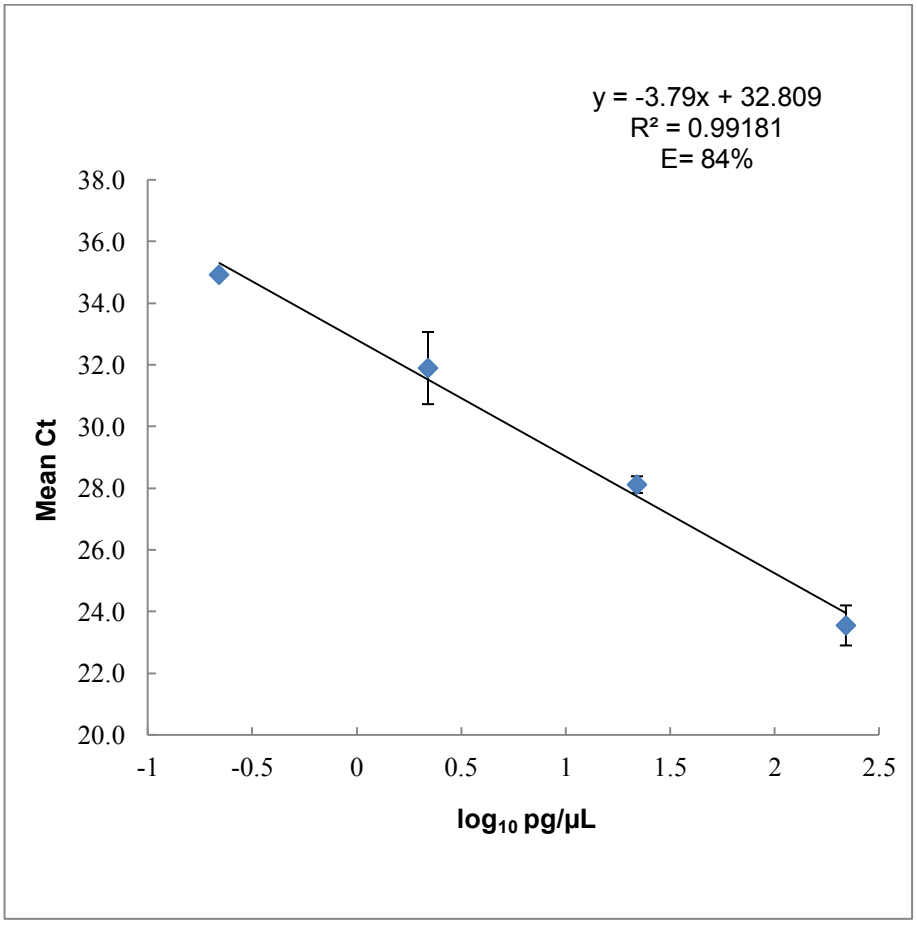

Figure 3.11 Neuregulin-1β standard curve from multiplex qPCR products of fibroblast mRNA extracts.

The fibroblast mRNA extraction and analytical procedures are as described in Figure 3.8. The extract was subjected to the multiplex qPCR at various dilutions and then analysed using a Cy5-labeled neuregulin-1β probe to ascertain the Ct values at each concentration of the extract. Each data point represents the mean ± SEM of triplicate determinations.

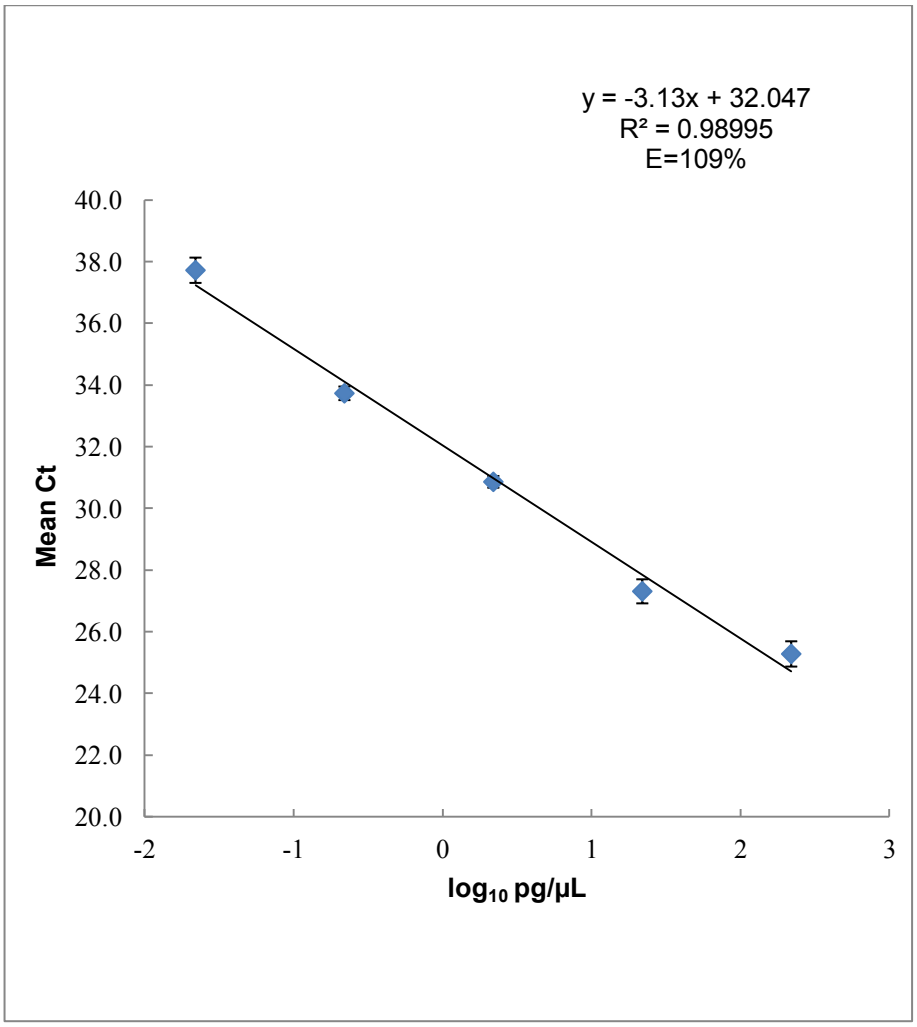

Figure 3.12 TGF-β standard curve from multiplex qPCR products of fibroblast mRNA extracts.

The fibroblast mRNA extraction and analytical procedures are as described in Figure 3.8. The extract was subjected to multiplex qPCR at various dilutions and then analysed using a HEX-labeled TGF-β probe to ascertain the Ct values at each concentration of the extract. Each data point represents the mean ± SEM of triplicate determinations.

irrespective of the duration of exposure. This conclusion is based on the observation that the relative amounts of neuregulin-1β and TGF-β mRNA, as determined by multiplex qPCR and quantified using the Pfaffl method (Pfaffl, 2001) were not significantly affected ($p > 0.05$; Tables 3.6 and 3.7). The large values for SEM in these two tables is almost certainly due to the fact that for both neuregulin-1β and TGF-1β the expression of is very much lower than that of GAPDH thus meaning that they are close to the limit of detection.

3.3.2 Effect of neuregulin-1β on β-adrenergic receptor gene expression

3.3.2.1 RT-PCR of β-adrenergic receptor mRNA in type II cell extracts in the presence and absence of RNase

The β-adrenergic receptor gene contains no introns and therefore no splice junctions in the corresponding mRNA (Emorine *et al.*, 1987; Kobilka *et al.*, 1987; Chatterjee *et al.*, 1997). Because of this, any products formed during RT-PCR of type II cell extracts could be generated from either mRNA or contaminating genomic DNA. In order to test whether there was DNA contamination of the type II cell extracts, RT-PCR was carried out using extracts previously incubated with and without RNase. When the RT-PCR products from type II cell extracts not treated with RNase were analyzed on an agarose gel (Figure 3.13), single PCR products of the appropriate size were observed for GAPDH (207 bp), Pgk (253 bp), Rpl13a (242 bp) and β-AR (152 bp), if the template was not previously treated with RNase. The transferrin receptor (Tfrc) gene appears not to be expressed in type II cells (Figure 3.13). If the template was incubated with 1.5-2 U RNase prior to RT-PCR no bands were observed using any of the primers. This demonstrates that the products observed in the absence of RNase were not the result of genomic DNA contamination of the template. Similar results were obtained with two separate RNA extracts.

Table 3.6 Expression of the neuregulin-1β gene in lung fibroblasts after exposure to dexamethasone.

Cultured fetal rat lung fibroblasts were exposed to MEM^+ containing 20 or 50 nM dexamethasone or an equivalent volume of propylene glycol (vehicle). After the indicated exposure time, the cells were subjected to RNA extraction and the mRNA concentration of these extracts quantified as described in 3.2.4.2 and 3.2.4.3, respectively. The level of neuregulin-1β mRNA was determined using multiplex qPCR, as described in 3.2.7. The data represent the mean ± SEM of the indicated number of independent fibroblast cultures.

Exposure time	**Neuregulin-1β gene expression in lung fibroblasts after exposure to dexamethasone** (relative to a control value of 1.00)	
	20 nM dexamethasone	**50 nM dexamethasone**
4 hours	0.93 ± 0.33 (5)	0.95 ± 0.41 (5)
6 hours	0.76 ± 0.12 (3)	0.58 ± 0.17 (3)
8 hours	0.89 ± 0.15 (6)	1.01 ± 0.35 (7)
24 hours	0.73 ± 0.42 (3)	1.10 ± 0.80 (3)

Table 3.7 Expression of the TGF-β gene in lung fibroblasts after their exposure to dexamethasone.

Cultured fetal rat lung fibroblasts were exposed to MEM^+ containing 20 or 50 nM dexamethasone or an equivalent volume of propylene glycol (vehicle). After the indicated exposure time, the cells were subjected to RNA extraction and the mRNA concentration of these extracts quantified as described in 3.2.4.2 and 3.2.4.3, respectively. The level of TGF-β mRNA was determined using multiplex qPCR, as described in 3.2.7. The data represent the mean ± SEM of the indicated number of independent fibroblast cultures.

Exposure time	TGF-β gene expression in lung fibroblasts after exposure to dexamethasone (relative to a control value of 1.00)	
	20 nM dexamethasone	**50 nM dexamethasone**
4 hours	0.99 ± 0.20 (5)	0.59 ± 0.15 (5)
6 hours	1.51 ± 0.47 (3)	1.00 ± 0.40 (3)
8 hours	0.47 ± 0.11 (5)	0.76 ± 0.32 (5)
24 hours	0.45 ± 0.24 (3)	0.47 ± 0.30 (3)

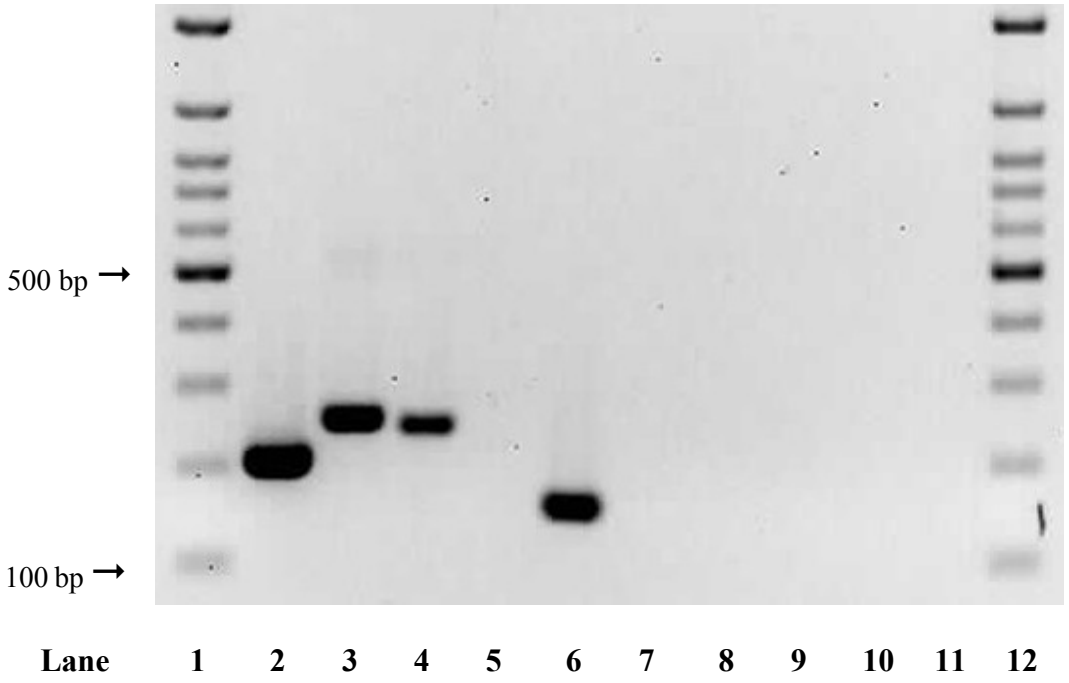

Figure 3.13 RT-PCR of the β-adrenergic receptor mRNA in type II cell extracts ± RNase.

RT-PCR was carried out using singleplex primers for GAPDH, Pgk, Rpl13a, Tfrc and β-adrenergic receptor (β-AR) and type II cell extracts before and after treatment with 1.5-2U RNase. An agarose gel was prepared and the RT-PCR products run as described in 3.2.8. *Lanes 1 and 12 - 100-3000 bp DNA ladder; lanes 2, 3, 4, 5 and 6 - GAPDH, Pgk, Rpl13a, Tfrc and β-AR (no RNase treatment of template), respectively; lanes 7, 8, 9, 10 and 11 - GAPDH, Pgk, Rpl13a, Tfrc and β-AR (after RNase treatment of template), respectively.*

3.3.2.2 Interaction between the RT-PCR primers for the β-adrenergic receptor and various reference genes

To determine which reference gene(s) would be most suitable in a multiplex PCR, an agarose gel was run of the RT-PCR products from RNA extracts of cultured rat lung type II cells using primers for GAPDH, Pgk, Rpl13a and β-AR, in singleplex and multiplex reactions. A single band was observed for each of the primer pairs, with the exception of those for Pgk, which had an additional faint band (Figure 3.14). For all multiplex incubations, except those of β-AR/GAPDH and β-AR/Pgk (lanes 8 and 9 of Figure 3.14), unexpected bands were apparent, indicating non-specific amplifications. Given that other researchers (Mak *et al.*, 1995a; Togaria *et al.*, 1997; Shi *et al.*, 2011) have used GAPDH as a reference gene with type II cells, all further studies were carried out using only GAPDH and β-AR in multiplex reactions. As three other potential reference genes, namely ACTB, Ppia and Tfrc, were shown not to be expressed at a sufficiently high level in type II cells, these were also deemed to be not suitable as reference genes.

3.3.2.3 Comparison of singleplex and multiplex RT-PCR products for both GAPDH and β-adrenergic receptor mRNA

When singleplex PCR reactions were carried out with either GAPDH or β-AR primers the RT-PCR products, when electrophoresed on an agarose gel, yielded single bands of the expected size (207 bp and 152 bp, respectively). When these same primers were used in a multiplex PCR reaction the same two bands were generated, however, the intensity of the smaller β-AR band was reduced (Figure 3.15). Importantly, no additional bands were generated when GAPDH or β-AR primers were used in this multiplex configuration, indicating that there weren't any non-specific amplifications.

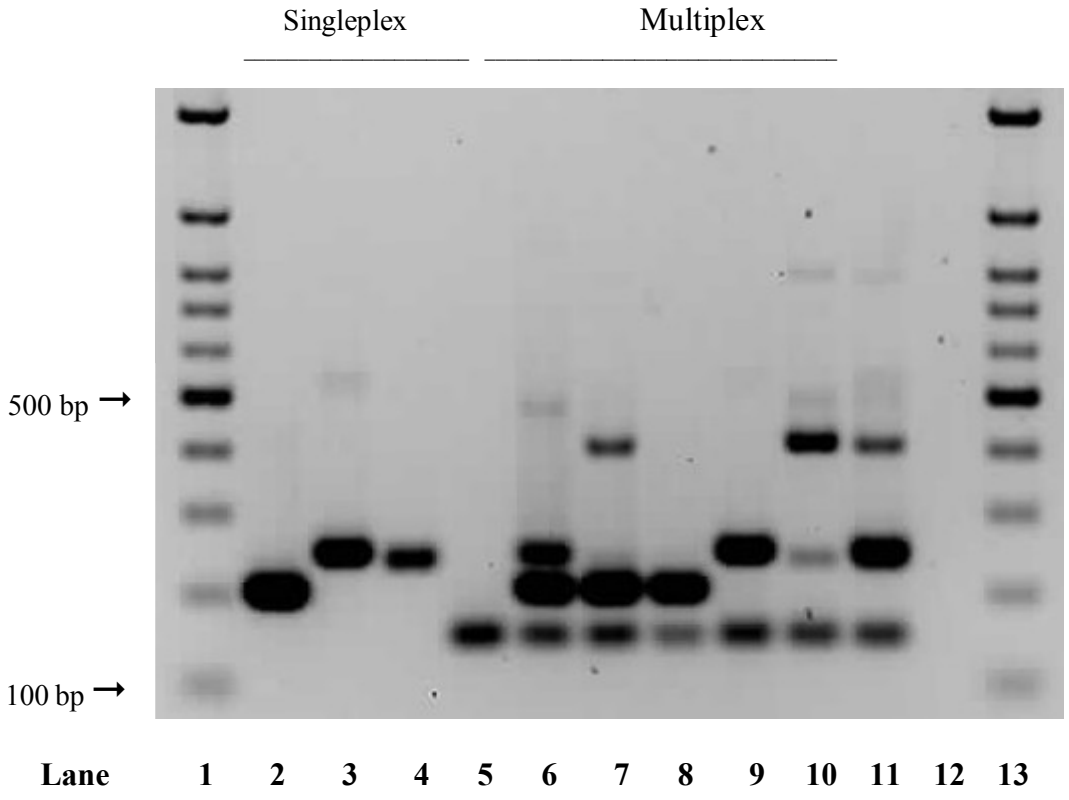

Figure 3.14 Test for any interaction between the RT-PCR primers for the β-adrenergic receptor and various reference genes.

RNA from cultured rat lung type II pneumocytes was extracted and quantified as described in 3.2.4.2 and 3.2.4.3, respectively. RT-PCR was carried out using singleplex and multiplex primers for GAPDH, Pgk, Rpl13a and β-AR. An agarose gel was prepared and the RT-PCR products ran as described in 3.2.8. *Lanes 1 and 13 - 100-3000 bp DNA ladder; lane 2 – singleplex GAPDH; lane 3 – singleplex Pgk; lane 4 – singleplex Rpl13a; lane 5 – singleplex β-AR; lane 6 - multiplex β-AR, Pgk and GAPDH; lane 7 - multiplex β-AR, Rpl13a and GAPDH; lane 8 - multiplex β-AR and GAPDH; lane 9 - multiplex β-AR and Pgk; lane 10 - multiplex β-AR and Rpl13a; lane 11 - multiplex β-AR, Rpl13a and Pgk; lane 12 – negative control (water).*

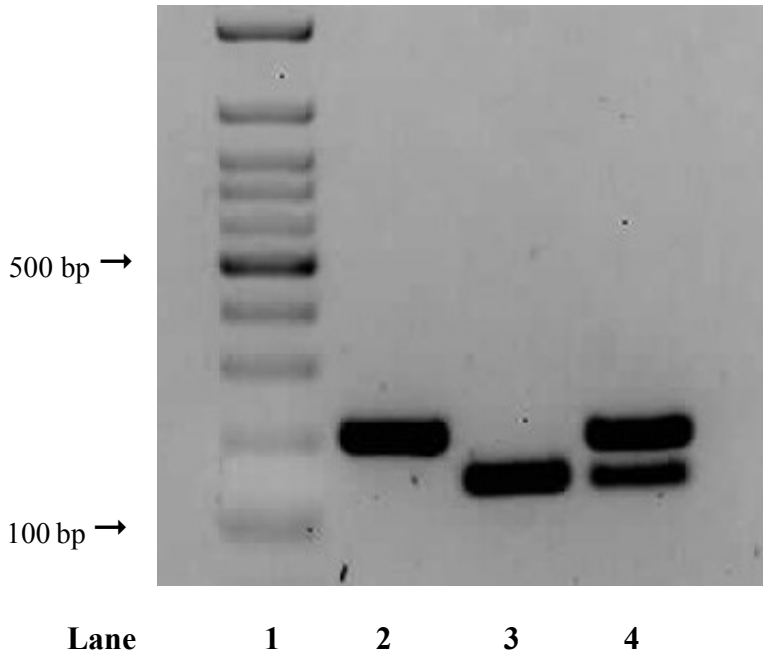

Figure 3.15 Gel of the singleplex and multiplex RT-PCR products for GAPDH and β-adrenergic receptor mRNA.

The type II cell mRNA extraction and analytical procedures are as described in Table 3.5. Singleplex and multiplex RT-PCR incubations were carried out using 1 μL of primers for both GAPDH and β-AR. An agarose gel was prepared and the RT-PCR products run as described in 3.2.8. *Lane 1 - 100-3000 bp DNA ladder; lane 2 - GAPDH; lane 3 - β-AR; lane 4 - multiplex incubation with both GAPDH and β-AR primers.*

In an attempt to minimize the reduction in the β-AR band, different relative concentrations of the GAPDH and β-AR primers were used. When the GAPDH primers were reduced to 0.05 μM and the β-AR primers increased to 0.4 μM (i.e. an 8-fold increase in the relative concentration of the latter) there was a decrease in the intensity of the GAPDH band and an increase in that of the β-AR band (Figure 3.16 – lane 3). When 0.1 μM of GAPDH and 0.4 μM β-AR primers were used (i.e. a 4-fold increase in the relative concentration of the latter), both GAPDH and β-AR bands were of high intensity. This latter relative ratio of the two primers was used in all subsequent experiments.

3.3.2.4 Standard curves for qPCR of type II cell mRNA extracts

Multiplex RT-PCR was carried out using primers and probes for GAPDH (FAM) and β-AR (HEX) with cultured rat lung type II cells RNA extracts either undiluted or diluted 10-, 100-, 1000- or 10,000-fold. Two bands were observed for GAPDH (207 bp) and β-AR (152 bp) after being run on an agarose gel. Because the RT-PCR was run for 40 cycles, a decrease in the intensity of the bands was only evident from about the 100-fold dilution (i.e. lane 8) of the template (Figure 3.17). Screen images using the QIAGEN Rotor-Gene Q 6000 software were captured to show the result for each of the probes - GAPDH (56-fam) and β-AR (5hex). The cycle threshold values (Ct) for each of GAPDH (FAM [green] channel) and β-AR (HEX [yellow] channel) increased as the template concentration decreased. For the template concentration range of 0.202-202.4 $pg.\mu L^{-1}$ the Ct values declined from 31.0-21.5 for GAPDH and from 36.5-26.4 for β-AR (Figure 3.18). Standard curves for GAPDH and β-AR were established by plotting the corresponding Ct values against the template concentration. Each curve revealed a linear relationship between these two parameters

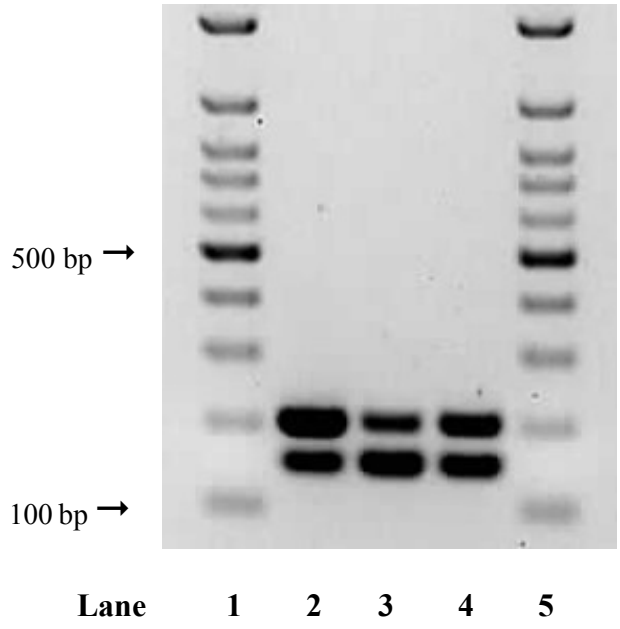

Figure 3.16 Effect of varying the concentrations of GAPDH and β-adrenergic receptor primers on the multiplex RT-PCR products.

The type II cell mRNA extraction and analytical procedures are as described in Table 3.5. RT-PCR was carried out using multiplex primers for GAPDH and β-AR at different concentrations of the primers. An agarose gel was prepared and the RT-PCR products run as described in 3.2.8. *Lanes 1 and 5 - 100-3000 bp DNA ladder; lane 2 – multiplex using 0.2 μM of both GAPDH and β-AR primers; lane 3 – multiplex using 0.05 μM of GAPDH and 0.4 μM of β-AR primers; lane 4 - multiplex using 0.1 μM of GAPDH and 0.4 μM of β-AR primers.*

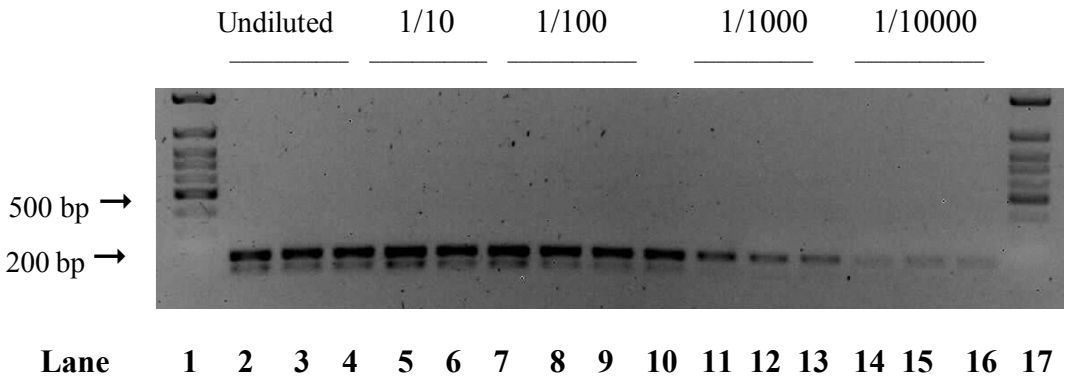

Figure 3.17 Gel of the multiplex (GAPDH/β-AR) PCR products of different concentrations of type II pneumocyte mRNA extracts.

RNA from cultured rat lung type II pneumocytes was extracted and quantified as described in 3.2.4.2 and 3.2.4.3, respectively. Multiplex RT-PCR was carried out in triplicate using GAPDH and β-AR primers at five different template concentrations. An agarose gel was prepared and the RT-PCR products run as described in 3.2.8. *Lanes 1 and 17 – 100-3000 bp DNA ladder; lanes 2, 3 and 4 – undiluted template; lanes 5, 6 and 7 - 1/10 diluted template; lanes 8, 9 and 10 - 1/100 diluted template; lanes 11, 12 and 13 - 1/1000 diluted template; lanes 14, 15 and 16 - 1/10000 diluted template.*

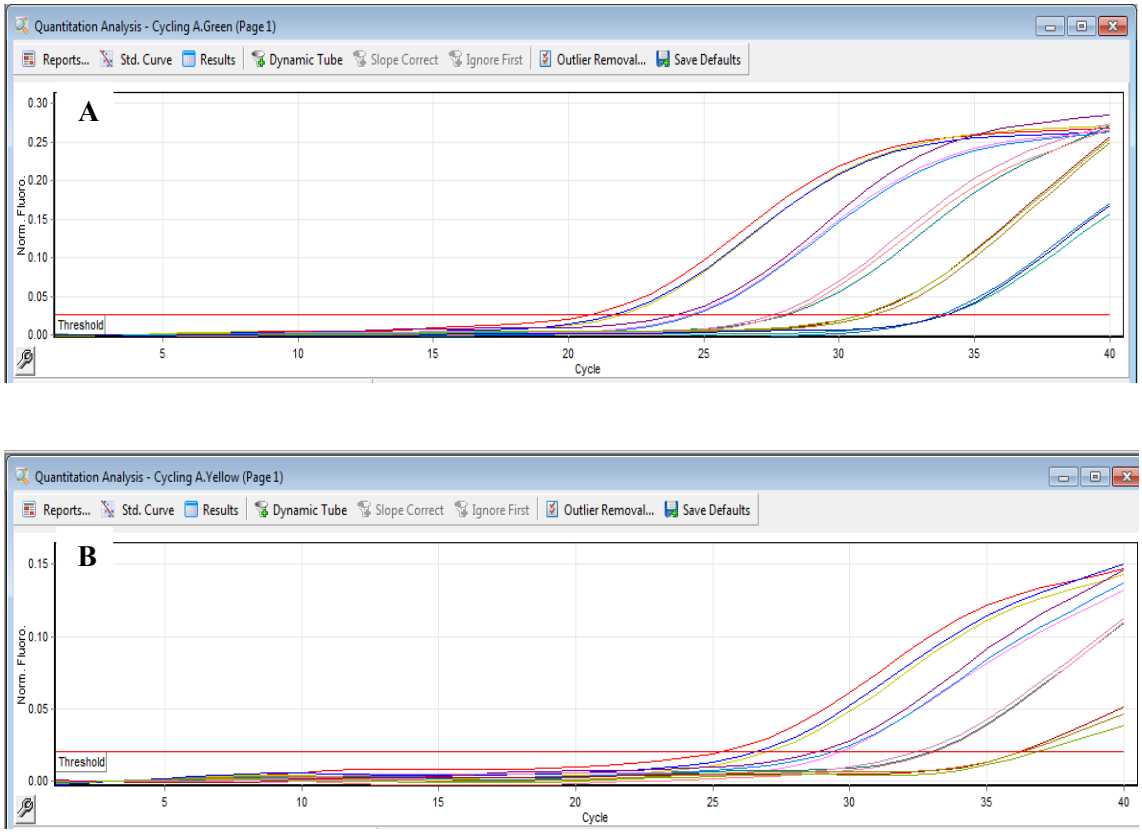

Figure 3.18 Amplification profiles of the GAPDH and β-AR standard curves.

The type II pneumocyte mRNA extraction and analytical procedures are as described in Figure 3.8. Screen images of the cDNA amplification profiles at five different template concentrations were captured using the (A) FAM (green), (B) HEX (yellow) channels to detect binding of the specific probes for GAPDH and β-AR, respectively.

($R^2 \geq 0.997$; see Figures 3.19 and 3.20). The efficiency (E) values were estimated to be 108% and 97% for GAPDH and β-AR, respectively.

3.3.2.5 Effects of neuregulin-1β and dexamethasone on β-adrenergic receptor gene expression in type II pneumocytes

Multiplex qPCR was carried out using both GAPDH and β-AR primers, together with the specific fluorescently-labeled TaqMan probes, for each of the qPCR products. RNA extracts were prepared from cultured type II pneumocytes that had been previously exposed to 20, 50 and 100 $ng.mL^{-1}$ heregulin-1β for 2 or 4 hours. These extracts were then subjected to multiplex qPCR and the level of β-AR gene expression was determined using the Pfaffl method (Pfaffl, 2001). There was no significant effect of the heregulin-1β on the level of β-AR gene expression when compared to that in the control extracts (Table 3.8).

A more extensive study was conducted in which the effects of dexamethasone (a known inducer of the β-adrenergic receptor) (Hadcock and Malbon, 1988; Cornett *et al.*, 1998) and heregulin-1β, alone and in combination, were determined. An approximate 2-fold increase in the expression of the β-AR gene ($p < 0.05$) was observed when type II cells were exposed to 50 nM dexamethasone for 6 hours (Figure 3.21). When the cells were exposed only to the indicated concentrations of heregulin-1β for the same duration, there was no significant effect on the level of β-AR mRNA. Moreover, when the cells were exposed to dexamethasone and heregulin-1β for 6 hours the level of β-AR mRNA was significantly different from that found in control cells ($p < 0.01$) but not significantly different ($p > 0.05$) from that in dexamethasone-treated cells.

When type II cells were likewise exposed to 50 nM dexamethasone for an extended period of 8 hours there was a 2-fold increase in the level of expression of the

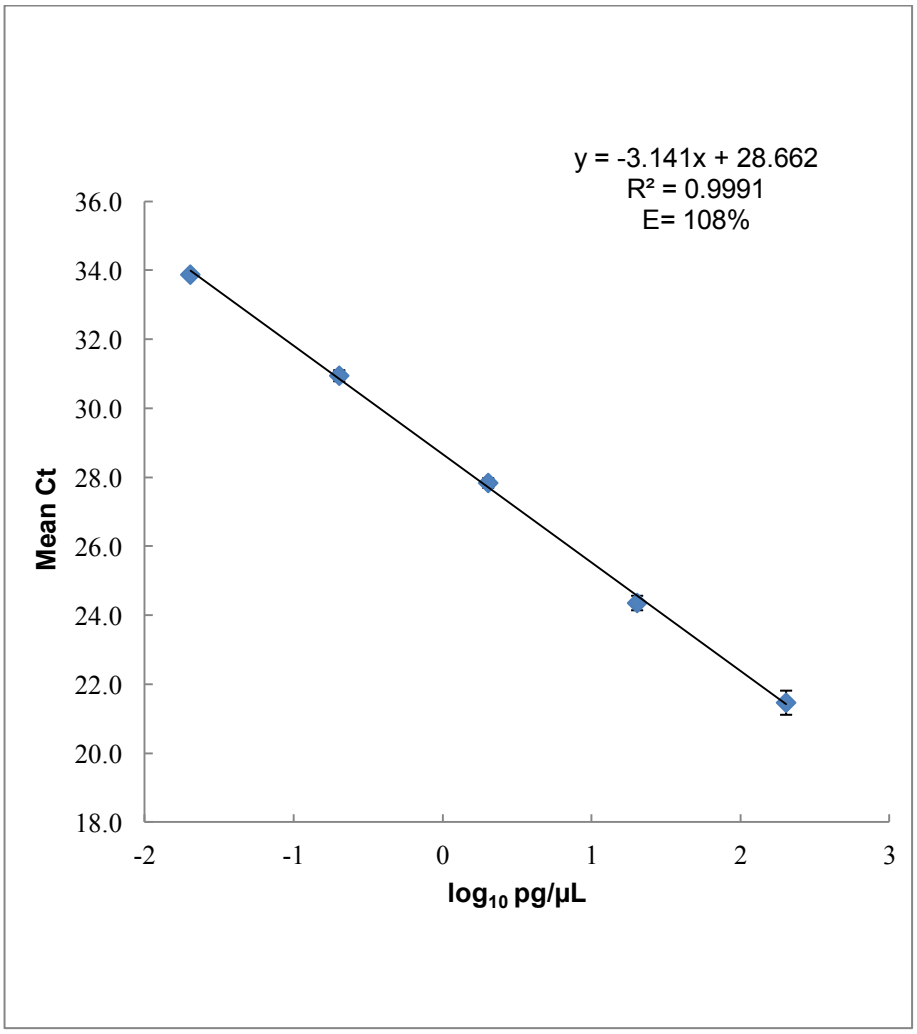

Figure 3.19 GAPDH standard curve from the multiplex qPCR products of the mRNA extracts from type II cells.

Type II cell mRNA extraction and analytical procedures are as described in Figure 3.18. The extract was subjected to multiplex qPCR at various dilutions and then analyzed using a FAM-labeled GAPDH probe to ascertain the Ct values at each concentration of the extract. Each data point represents the mean ± SEM of triplicate determinations.

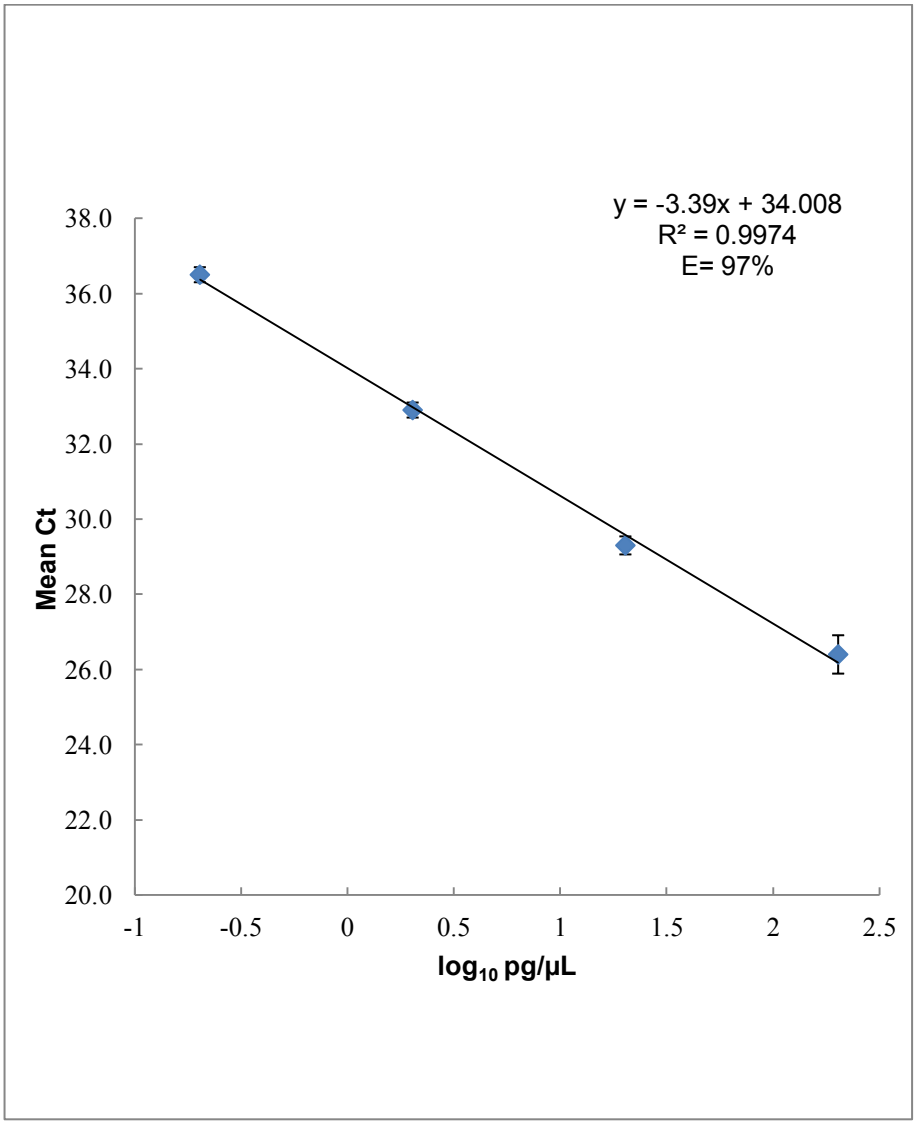

Figure 3.20 β-adrenergic receptor standard curve from the multiplex qPCR products of the mRNA extracts from type II cells.

Type II cell mRNA extraction and analytical procedures are as described in Figure 3.18. The extract was subjected to multiplex qPCR at various dilutions and then analyzed using a HEX-labeled β-adrenergic receptor probe to ascertain the Ct values at each concentration of the extract. Each data point represents the mean ± SEM of triplicate determinations.

Table 3.8 Expression of the β-adrenergic receptor gene in lung type II cells after exposure to heregulin-1β.

Rat type II cells were isolated from fetal lung tissue and grown in culture for 3 days prior to exposing them to MEM^+ containing 20, 50 or 100 $ng.mL^{-1}$ of heregulin-1β or an equivalent volume of water (control). After the indicated exposure times, the cells were subjected to RNA extraction and the mRNA concentration of these extracts quantified as described in 3.2.4.2 and 3.2.4.3, respectively. The level of β-AR mRNA was determined using qPCR, as described in 3.2.7. The data represent the mean ± SEM of the specified number of independent type II cells cultures.

Exposure time	**β-adrenergic receptor gene expression in lung type II cells after exposure to heregulin-1β** (relative to a control value of 1.00)		
	20 $ng.mL^{-1}$	**50 $ng.mL^{-1}$**	**100 $ng.mL^{-1}$**
2 hours	1.28 ± 0.03 (4)	0.80 ± 0.16 (6)	0.97 ± 0.43 (4)
4 hours	1.06 ± 0.13 (4)	0.99 ± 0.08 (6)	0.94 ± 0.24 (4)

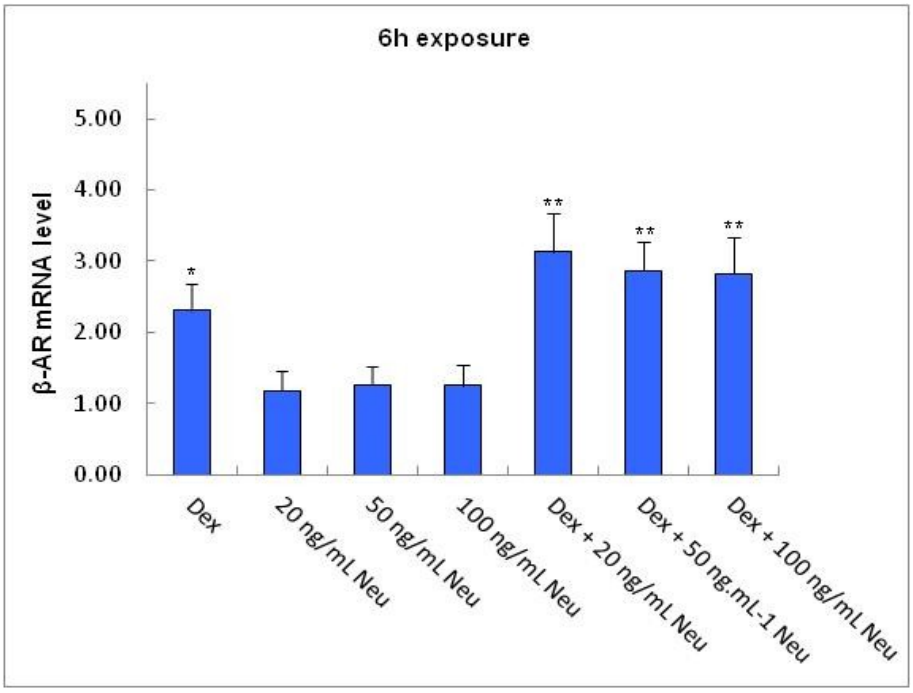

Figure 3.21 Effect of neuregulin-1β on β-adrenergic receptor gene expression in type II pneumocyte after 6 h exposure.

Rat type II cells were isolated from fetal lung tissue and grown in culture for 3 days prior to exposing them to MEM^{+} containing 20, 50 or 100 $ng.mL^{-1}$ of heregulin-1β or an equivalent volume of water (control) with or without 50 nM dexamethasone. After 6 h of further incubation, the cells were subjected to RNA extraction and the mRNA concentration of these extracts quantified as described in 3.2.4.2 and 3.2.4.3, respectively. The level of β-AR mRNA was determined using multiplex qPCR with both GAPDH and β-AR primers, as described in 3.2.7. The results, which represent the level of β-AR gene expression relative to that in control cells, are the mean ± SEM of 5 separate experiments conducted in duplicate. Significant differences from the level of expression in control cells are indicated with asterisks (∗ $p < 0.05$, ∗∗ $p < 0.01$).

β-AR gene. However, although there was no significant effect of neuregulin-1β alone, the β-AR mRNA level was significantly greater when the cells were exposed to either 50 or 100 $ng.mL^{-1}$ heregulin-1β, in combination with dexamethasone, when compared to steroid treatment alone ($p < 0.05$ and $p < 0.001$, respectively). This indicates that dexamethasone and heregulin-1β act synergistically if the exposure time is at least 8 hours (Figure 3.22).

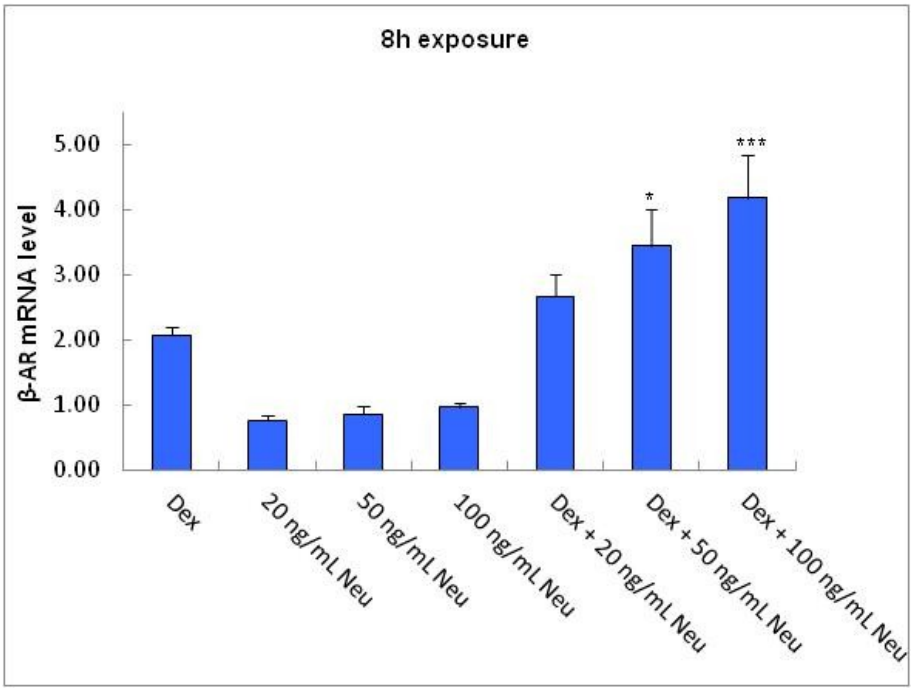

Figure 3.22 Effect of neuregulin-1β on β-adrenergic receptor gene expression in type II pneumocyte after 8 h exposure.

Rat type II cells were isolated from fetal lung tissue and grown in culture for 3 days prior to exposing them to MEM^{+} containing 20, 50 or 100 $ng.mL^{-1}$ of heregulin-1β or an equivalent volume of water (control) with or without 50 nM dexamethasone. After 8 h of further incubation, the cells were subjected to RNA extraction and the mRNA concentration of these extracts quantified as described in 3.2.4.2 and 3.2.4.3, respectively. The level of β-AR mRNA was determined using multiplex qPCR with both GAPDH and β-AR primers, as described in 3.2.7. The results, which represent the level of β-AR gene expression relative to that in control cells, are the mean ± SEM of 5 separate experiments conducted in duplicate. Significant differences from the level of expression in those cells exposed only to dexamethasone are indicated by asterisks (* $p < 0.05$; *** $p < 0.001$).

3.4 Discussion

3.4.1 Effect of dexamethasone on neuregulin-1β gene expression

Before ascertaining the effect of glucocorticoids on neuregulin-1β gene expression in lung fibroblasts several tests were conducted to confirm that, during RT-PCR, there was no interaction between the primers or probes for GAPDH (reference gene), neuregulin-1β (target gene) and TGF-β, a gene reported to be attenuated by glucocorticoid treatment (McDevitt *et al.*, 2007). In addition, standard curves were established for each of these gene products.

Primers, together with mRNA extracts from lung fibroblasts from day 19 fetal rats, were run as separate and multiplex RT-PCR incubations. Upon completion of the RT-PCR, samples were subjected to electrophoresis on agarose gels, which confirmed that the products observed were the anticipated size for each of the primer pairs used (i.e. GAPDH 207 bp; neuregulin-1β 196 bp and TGF-β 392 bp) and indicated that, under singleplex RT-PCR incubation conditions, the products contained only those that were expected. Under multiplex conditions only the expected bands were generated which demonstrates that there was no interference between the various primers and probes.

When the mRNA template was increasingly diluted and RT-PCR carried out followed by a separation of the products on an agarose gel, a decrease in the intensity of the bands was only evident at the two highest levels of dilution. Screen capture images of the GAPDH (56-fam), neuregulin-1β (5cy5) and TGF-β (5hex) products on the appropriate channels showed an increase in the cycle threshold values (Ct) as the template concentration decreased. Standard curves were established by plotting these Ct values against the template concentration thus providing an efficiency value (E) for each gene product. The efficiency values for GAPDH, neuregulin-1β and TGF-β were 113%,

84% and 109%, respectively. The Ct values for both test (20 and 50 nM dexamethasone) and control conditions, together with the efficiency values, were used in the determination of neuregulin-1β and TGF-β gene expression using the method of Pfaffl (2001), which calculates the relative expression of the target genes in comparison to that of the housekeeping gene GAPDH. Irrespective of the concentration of dexamethasone used, or the time of exposure to the steroid, there was neither a significant enhancement nor suppression in the expression of the neuregulin-1β or TGF-β genes, respectively. This shows that the higher level of neuregulin-1β produced by lung fibroblasts in the presence of dexamethasone (Maker, 2008) was not the result of enhanced neuregulin-1β gene expression. Given that neuregulins are produced as transmembrane precursors, which are considered to generate diffusible ligands when subjected to cleavage (Crovello *et al.*, 1998), it is possible that dexamethasone stimulates the rate of cleavage of the neuregulin precursors. Such a cleavage of membrane-bound pro-NRG protein leading to the release of soluble neuregulin-1β has been show to occur in neuronal cells when activation of protein kinase C by neurotrophin results in the phosphorylation of the membrane-bound, precursor protein (Esper and Loeb, 2009). Other possibilities are raised in the General Discussion.

The failure of dexamethasone to significantly reduce the expression of TGF-β in rat lung fibroblasts (see Table 3.7) is in contrast to the observation of McDevitt *et al.* (2007), who showed that the level of TGF-β in fetal human lung epithelial cells was diminished when the cells were exposed to dexamethasone. Given that both rat lung fibroblasts and type II cells contain an almost identical concentration of the glucocorticoid receptor (Damas, 2010), the contradiction referred to above is thus likely to be due to the TGF-β gene of fetal human lung epithelial cells and rat lung fibroblasts being differently affected by glucocorticoids.

3.4.2 Effect of neuregulin-1β on β-AR gene expression

Because β-AR gene has no introns (Emorine *et al.*, 1987; Kobilka *et al.*, 1987; Chatterjee *et al.*, 1997), an experiment was carried out with and without RNase treatment of the template. It was established that pre-treatment of the template with RNase eliminated the formation of any RT-PCR product using primers not only for β-AR but also for Pgk, Rpl13a and GAPDH. This confirms that the DNase, present during the RNA extraction, was sufficient to remove any contaminating genomic DNA and that any product formed was derived from extracted cellular RNA.

Additional experiments showed that the transferin receptor (Tfrc) gene was not expressed in type II cells and that there was an undesirable interaction between the primers for β-AR and Rpl13a in a multiplex incubation (Figure 3.14). This renders both Tfrc and Rpl13a unsuitable candidates to act as the reference gene. In contrast, both GAPDH and Pgk met all the requirements for this role including no sign of any interactions between the primers for these reference genes and those of the β-AR gene in a multiplex incubation. Given that other researchers (Mak *et al.*, 1995a; Togari *et al.*, 1997; Togaria *et al.*, 1997; Shi *et al.*, 2011) have used GAPDH as the reference gene for type II cells, all of the studies reported here were carried out using only GAPDH and β-AR primers in multiplex reactions. Initial experiments gave a diminished signal for the β-AR product when both GAPDH and β-AR primers were present in a multiplex reaction. This problem was solved by changing the relative concentrations of the two sets of primers. When GAPDH primers were reduced to 0.1 μM and the concentration of the β-AR primers increased to 0.4 μM both GAPDH and β-AR bands were of high intensity. This latter relative ratio of the two primers was used in all further experiments.

When the mRNA template was serially diluted as described in Figure 3.17, subjected to RT-PCR and the products analysed using agarose gels, the intensity of the bands showed a progressive decrease from a 100-fold dilution of the template. The results presented in the screen capture images of the GAPDH and β-AR qPCR products in the 56-fam (green) and 5hex (yellow) channels, respectively, showed increasing cycle threshold values (Ct) as the template concentration decreased. Standard curves were established by plotting the Ct values against the template concentration to provide the efficiency values (E) for each gene product. The efficiency values for GAPDH and β-AR were 108% and 97%.

The Ct values for each of the test (20, 50 and 100 $ng.mL^{-1}$ heregulin-1β) and control conditions, together with the efficiency values, were used in conjunction with the method of Pfaffl (2001) to determine the impact of heregulin-1β on the extent of β-AR gene expression in type II pneumocytes. Irrespective of whether the cells were exposed to heregulin-1β for 2, 4, 6 or 8 hours there was no increase in the expression of the β-AR gene (Table 3.8; Figures 3.21 and 3.22). In contrast, if the type II cells were incubated with 50 nM dexamethasone for 6 hours, there was a significant increase ($p < 0.05$) in the level of expression of the β-AR gene as compared to control cultures. This is in agreement with numerous investigators who have shown similar levels of induction of the β-AR gene after glucocorticoid administration in a variety of tissues (Collins *et al.*, 1988; Hadcock and Malbon, 1988; Hadcock *et al.*, 1989; Mak *et al.*, 1995a; Dangel *et al.*, 1996; Cornett *et al.*, 1998), including the lung (Mak *et al.*, 1995a; Cornett *et al.*, 1998). Although the expression of the β-AR gene was also elevated when type II cells were exposed to 20, 50 and 100 $ng.mL^{-1}$ heregulin-1β in the presence of 50 nM dexamethasone ($p < 0.01$), this was not significantly different from that which occurred with exposure to dexamethasone alone.

However, when the type II cells were exposed to these same agents for an extended period of 8 hours, although a similar level of induction of the β-AR gene occurred when the cells were exposed only to dexamethasone, and there was no effect of heregulin-1β alone, there was a much greater level of induction if the cells were exposed to this peptide in the presence of 50 nM dexamethasone (Figure 3.22). This enhanced level of induction was only significant when the heregulin-1β concentration was 50 or 100 $ng.mL^{-1}$ (3.4-fold; $p < 0.05$ and 4.2-fold; $p < 0.01$, respectively, as compared to the expression level in cells exposed to dexamethasone alone, i.e. 2.1-fold). This is the first time that dexamethasone and heregulin-1β have been shown to act synergistically in inducing the expression of the β-AR gene. Damas (2010) has shown that, although dexamethasone when applied directly to type II cells increases the β-AR activity, the response to this steroid was significantly greater if the type II cells were exposed to FCM generated in the presence of dexamethasone (see Figure 3.23). This could be explained if the preconditioning of the media by fibroblasts in the presence of dexamethasone leads not only to the carryover of the steroid, but also the generation of neuregulin-1β leading to a synergistic response.

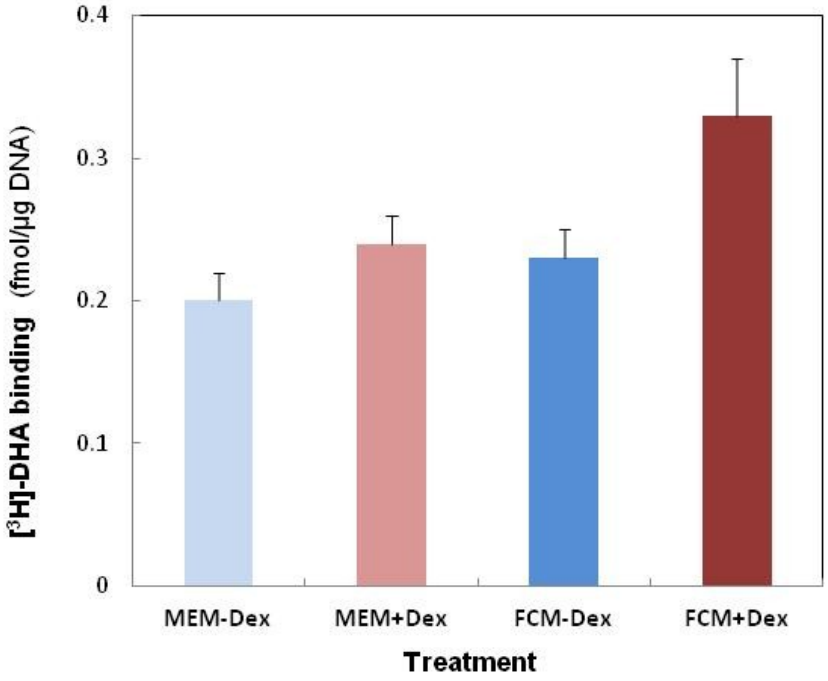

Figure 3.23 Effect of dexamethasone on β-adrenergic receptor activity in cultured fetal rat type II cells.

Rat type II pneumocytes were isolated from fetal lung tissue and grown in 24–well tissue culture plates for 3 days as described in 2.2.4. After being washed thoroughly using BSS, cells were then incubated for an additional 24 h in either MEM^{+} or fibroblast-conditioned media with (■ ■) and without 100 nM dexamethasone (■ ■), respectively. The cells were then tested for specific β-AR activity as described in 2.2.8. The results represent the mean ± SEM of three separate experiments conducted in duplicate. This figure is included with the approval of the author, Jolanta Damas (Damas, 2010).

Chapter 4

General discussion

4.1 Effect of heregulin-1β on surfactant phospholipid synthesis in type II cells

Although glucocorticoids are known to stimulate the maturation of the lung through enhanced surfactant phospholipid synthesis, they have no direct effect on type II cells (Smith, 1978; Maker, 2008). However, when lung fibroblasts are cultured in the presence of dexamethasone, the resultant fibroblast-conditioned media (FCM) has been shown to have a stimulatory effect on surfactant production in cultured type II cells (Smith, 1978). This indirect effect of glucocorticoids upon surfactant phospholipid synthesis was attributed to a fibroblast-derived peptide, termed fibroblast-pneumocyte factor (FPF) (Smith, 1979). It was subsequently reported that purified FPF was able to directly stimulate the synthesis of surfactant-associated phospholipids (Post and Smith, 1984), which resulted from a significant elevation in the activity of choline phosphate cytidylyltransferase (Post *et al.*, 1986). Moreover, a monoclonal antibody raised against FPF blocked glucocorticoid-induced maturation of the lung (Post *et al.*, 1984). Despite numerous attempts to identify FPF (Torday and Kourembanas, 1990; Torday *et al.*, 2002; Dammann *et al.*, 2003; Maker, 2008), its chemical nature is still not certain. Subsequent to the finding of Dammann *et al.* (2003) that the stimulatory effect of FCM could be mimicked by neuregulin-1β and blocked by a neuregulin-1β antibody, Maker (2008) showed that FCM generated in the presence of dexamethasone had an elevated concentration of neuregulin-1β. Together these findings suggest that neuregulin-1β might be, at least, a major component of FPF.

The current study has shown that surfactant phospholipid synthesis was elevated when type II cells were exposed to the recombinant form of neuregulin-1β, heregulin-1β (Figures 2.4 and 2.5). This effect was maximal at a heregulin-1β concentration of 20 $ng.mL^{-1}$ but was not evident at 100 and 200 $ng.mL^{-1}$. It is suggested

that these higher concentrations of the peptide lead to a downregulation of the ErbB receptors to which heregulin-1β binds. Such a conclusion is compatible with the observations of Cao *et al.* (2007) that, in MCF-7 breast cancer cells, neuregulin-1 stimulates both ubiquitination and degradation of the ErbB3 receptor leading to lower levels of the receptor when the cells are exposed to high levels of the ligand.

The stimulation of surfactant phospholipid synthesis by heregulin-1β in cultured fetal type II cells is in agreement with the findings of Dammann *et al.* (2003) that implicate neuregulin-1β as a mediator of the indirect effect of glucocorticoids on these cells. It is also compatible with the observation of Maker (2008) that exposure of rat lung fibroblasts to dexamethasone led to an elevation in the media concentration of neuregulin-1β. However, there was no change in the relative expression of the neuregulin-1β gene in response to dexamethasone (Table 3.6), irrespective of the concentration or the time of exposure to this steroid. This shows that the higher concentration of neuregulin-1β in the media of lung fibroblasts exposed to dexamethasone (Maker, 2008) is not the result of enhanced neuregulin-1β gene expression. Given that neuregulins are formed as transmembrane precursors, which give rise to diffusible ligands when subjected to cleavage (Crovello *et al.*, 1998), it is possible that dexamethasone stimulates the rate of cleavage of the neuregulin precursors. In this context it is relevant that the growth factor neurotrophin stimulates the activation of the δ-isoform of protein kinase C in neuronal cells thereby causing phosphorylation and cleavage of the membrane-bound pro-NRG1 protein, thus releasing soluble neuregulin-1β (Esper and Loeb, 2009). Alternatively, the effect of dexamethasone could be the result of enhanced mRNA or protein stability or the result of an elevated rate of mRNA translation.

McDevitt *et al.* (2007) have previously shown that dexamethasone treatment reduces the expression of the TGF-β gene in cultured fetal human type II cells. It was reasoned that, if such a decline in TGF-β gene expression also occurred in fetal rat lung fibroblasts, this would be a useful indicator of a dexamethasone response in these cells. Although there did appear to be a decrease in the level of expression of the TGF-β gene in cultured fibroblasts exposed to dexamethasone, particularly after longer exposure times, these differences were not statistically significant (Table 3.7). The influence of glucocorticoids on lung maturation could be partly accounted for by a decrease in the concentration of TGF-β, which has been previously shown to inhibit early morphogenesis and block hormone-induced type II cell differentiation (Torday and Kourembanas, 1990; McDevitt *et al.*, 2007). Thus the effects of glucocorticoids in regulating surfactant production are likely to involve both elevated levels of stimulatory factors and diminished levels of inhibitory factors.

Leptin, which has a molecular weight within the range previously reported for FPF (Smith, 1979), has been shown to stimulate surfactant phospholipid synthesis by fetal type II pneumocytes in a concentration-dependant manner (Torday and Rehan, 2002; Torday *et al.*, 2002). Such a finding has been confirmed in the current study (Figure 2.6), which showed that leptin was more potent than heregulin-1β (after 4 h exposure) in stimulating phospholipid synthesis. The expression of the leptin gene in lung fibroblasts has been shown to progressively increase during the latter stages of gestation and to be enhanced if these cells are exposed to dexamethasone or parathyroid hormone-related protein (PTHrP) (Torday *et al.*, 2002). These authors concluded that, based on their similarities, leptin and FPF share a common identity. Subsequently, Torday and Rehan (2002) reported that the stretch-induced stimulation of surfactant synthesis, a response that is only evident when type II pneumocytes and lung fibroblasts are co-cultured, is mediated by PTHrP, leptin and their corresponding receptors.

However, in a recent publication, Huang *et al.* (2012) demonstrated that deletion of ErbB1 or downregulation of ErbB4 prevented stretch-induced type II cell differentiation. This finding, together with the fact that leptin interacts with the leptin (Ob-Rb) receptor and not the ErbB receptors, suggests that factors other than leptin might be involved in this response. Moreover, stretch-induced stimulation of lung differentiation has been shown to be associated with an elevation in the concentration of prostanoids (Copland *et al.*, 2006) and various cytokines (Copland and Post, 2007).

Although leptin has been proposed as a likely candidate for the role of FPF in lung development (Torday *et al.*, 2002), the more recent findings of Dammann *el al.* (2003) together with the observations presented in this thesis suggest that FPF may actually be a complex mixture including leptin, neuregulin-1β and other regulatory peptides (Copland *et al.*, 2006; Copland and Post, 2007). These various agents could account for the range of effects which lead to the development and maturation of the lung.

4.2 Surfactant phospholipid secretion from type II cells.

Exposure of type II cells to heregulin-1β resulted in a concentration- and time-dependent stimulation of surfactant phospholipid secretion from these cells. After a three-hour treatment of the cells with 50 $ng.mL^{-1}$ heregulin-1β, there was a 2.4-fold increase in the amount of phospholipids secreted (Figure 2.8). When type II cells were treated with similar concentrations of leptin, a peptide known to stimulate surfactant synthesis (Figure 2.6) (Torday and Rehan, 2002; Torday *et al.*, 2002; Kirwin *et al.*, 2006), there was no effect on the rate of surfactant secretion from these cells. This contrasts with the finding that 50 $ng.mL^{-1}$ heregulin-1β not only stimulates surfactant phospholipid synthesis but also enhances its secretion (Figures 2.5 and 2.8).

It has been known for many years that β-agonists, operating through an interaction with the β-adrenergic receptor, markedly stimulate the secretion of surfactant phospholipids from type II cells (Dobbs and Mason, 1979; Brown and Longmore, 1981). However, long-term use of β-agonists would need to be undertaken with caution as Lin *et al.* (2012) have recently shown that β-AR dysfunction can result from repeated clinical use of β-agonists in the treatment of asthma. A previous study from this laboratory has shown that 24-hour exposure of cultured fetal type II cells to heregulin-1β resulted in a significant elevation in the level of the membrane-bound β-AR (Figure 2.10). It is known that there are two major subtypes of β-adrenergic receptor, β_1 and β_2 subtypes, and both have been shown to coexist in mammalian lung (Rugg *et al.*, 1978). However, subsequent studies concluded that for fetal rat type II pneumocytes (Fabisiak *et al.*, 1987), whole fetal rat lung (Whitsett *et al.*, 1981) and fetal human lung (Davis *et al.*, 1987) the predominant receptor is the β_2 subtype. Irrespective of whether the type II cells were derived from male or female rat fetuses, exposure of these cells to heregulin-1β resulted in a significant elevation in the level of membrane-bound β-adrenergic receptors (Figure 2.11).

As the response to β-agonists in stimulating surfactant phospholipid secretion from type II pneumocyte is known to be influenced by the level of β-AR it is of interest that media, which had been conditioned by fetal rat fibroblasts in the presence of dexamethasone, enhanced the β-AR activity to a greater extent if the cells were derived from female fetuses (Damas, 2010). This implies that female-derived fibroblasts produce a higher concentration of FPF than the equivalent cells derived from male fetal rats. As the heregulin-1β-induced activity of β-AR is virtually identical in cells derived from either sex, it suggests that this sex-linked difference is an attribute of lung fibroblasts, which is consistent with the previously published conclusions of Torday (1984) and Floros *et al.* (1987).

In the current study the higher level of β-AR induced by heregulin-1β has been shown to result in a greater sensitivity of the type II cells to the β-adrenergic agonist (—)-isoproterenol. The finding that this agonist only slightly elevated the rate of surfactant phospholipid secretion from control type II cells is in contrast to an earlier study by Dobbs and Mason (1979). This may be explained by the fact that in their study culture medium was supplemented with 10% fetal calf serum, which is known to contain glucocorticoids, whereas in the current investigation steroids were removed by pre-treating the serum with charcoal. However, the response to (—)-isoproterenol was markedly increased when these cells were pre-treated with heregulin-1β (Figure 2.13). Although it has been previously shown that (—)-isoproterenol stimulates surfactant phospholipid secretion from epithelial cells (Dobbs and Mason, 1979; Ormond *et al.*, 2003; Abraham *et al.*, 2004; Abrahama *et al.*, 2004; Shi *et al.*, 2011), this study, to my knowledge, is the first to show that the response to (—)-isoproterenol is amplified by pre-treatment with heregulin-1β. This heregulin-1β-induced elevation in the response to (—)-isoproterenol is also unaffected by the sex of the fetuses from which the type II cells were derived (Figure 2.14). Thus, neither the induction of β-AR by heregulin-1β nor its enhancement of β-agonist-induced secretion of surfactant phospholipids from fetal type II pneumocytes is affected by the sex of the donor animal.

4.3 Effect of neuregulin-1β on β-AR gene expression

In the concentration range of 20-100 ng.mL^{-1}, heregulin-1β had no impact on the level of expression of the β-AR gene in type II cells (Table 3.8; Figures. 3.21 and 3.22), irrespective of whether the cells were exposed to heregulin-1β for 2, 4, 6 or 8 hours. In contrast, if the type II cells were incubated with 50 nM dexamethasone for 6 hours, there was a significantly greater level of expression of the β-AR gene ($p < 0.05$) when compared to the level of expression in control cultures. This verifies the findings of a number of investigators who have shown that exposure to glucocorticoids induces

the β-AR gene in a variety of tissues (Collins *et al.*, 1988; Hadcock and Malbon, 1988; Hadcock *et al.*, 1989; Mak *et al.*, 1995a; Dangel *et al.*, 1996; Cornett *et al.*, 1998), including the lung (Mak *et al.*, 1995a; Cornett *et al.*, 1998). When type II cells were exposed to the combined effects of heregulin-1β and dexamethasone for 6 hours, although the level of expression of the β-AR gene was slightly higher than that resulting from exposure to the steroid alone, this difference was not significant (Figure 3.21). In contrast, when the cells were simultaneously exposed to these two agents for a period of 8 hours, there was a significantly higher level of β-AR gene expression than that which occurred in response to dexamethasone alone (Figure 3.22). This is the first time that dexamethasone and heregulin-1β have been shown to act synergistically in inducing the expression of the β-AR gene. This latter finding may provide an explanation for the previously mentioned observation of Damas (2010) that the elevation of β-AR activity was greater in type II cells exposed to FCM generated in the presence of dexamethasone than in cells directly exposed to the same concentration of the steroid (Figure 3.23). This could be explained if the preconditioning of the media by fibroblasts in the presence of dexamethasone leads not only to the carryover of the steroid, but also the generation of neuregulin-1β leading to a synergistic response. This further supports the hypothesis that neuregulin-1β is a component of FPF.

An anomalous result was the finding that exposure of type II cells to heregulin-1β for 24 hours enhanced the β-AR activity (Figure 2.11) despite this treatment having no effect on the level of β-AR gene expression at 2, 4 or 8 hours (Table 3.8, Figures 3.21 and 3.22). There are at least two possibilities that could account for this apparently contradictory observation. The up-regulation of β-AR activity by heregulin-1β may either be via a non-genomic mechanism or the expression of the β-AR gene could be transiently elevated in response to heregulin-1β at some time after 8 h exposure, leading to an enhanced activity of β-AR at 24 h. This latter suggestion is

consistent with the observations that dexamethasone induces a transient elevation in the β-AR mRNA at 2 hours but an enhanced β-AR activity 22 hours later in adult lung tissue from both humans (Mak *et al.*, 1995a) and rats (McGraw *et al.*, 1995).

4.4 Suggestions for future work

The concentrations of both leptin and neuregulin-1β have been shown to be elevated in FCM in response to glucocorticoids. Moreover, each of these peptides stimulates surfactant phospholipid synthesis. As a consequence, it is difficult to know which of these peptides is the active component of FCM. This issue could be clarified by using specific antibodies raised against leptin and neuregulin-1β.

The finding that neuregulin-1β concentration is elevated in FCM when the fibroblasts are exposed to glucocorticoids despite there being no effect of the steroid on neuregulin-1β gene expression appears to be anomalous. It has been suggested that the glucocorticoid-induced increase in the concentration of neuregulin-1β could be the result of enhanced cleavage of membrane-bound neuregulin precursors, enhanced mRNA or peptide stability or an elevation in the rate of mRNA translation. Additional experiments need to be conducted to ascertain which of these possibilities is the actual mechanism.

Since the β-AR receptor activity in type II cells was induced by a similar magnitude in cells derived from either male or female fetuses, it was assumed that the sex-linked difference in the response of these cells to glucocorticoids resided with the lung fibroblasts. It has previously been shown that exposure of lung fibroblasts to glucocorticoids elevated the level of neuregulin-1β in the surrounding media (Maker, 2008), however, it should be noted that in that study the fibroblasts were derived from a mixture of both male and female fetuses. Given that many of the effects of glucocorticoids upon type II cells are suggested in this thesis as being mediated by

neuregulin-1β, it is important that the impact of glucocorticoids on the production and/or release of neuregulin-1β by lung fibroblasts be examined in cells derived from either male or female fetuses.

Although heregulin-1β has no effect on β-AR gene expression alone, it acts synergistically with dexamethasone to further enhance the level of expression of this gene when type II cells were exposed simultaneously to these two agents (Figure 3.22). In contrast, a previous preliminary investigation in this laboratory (Liong Ka Hang's Honours thesis) showed that both heregulin-1β and dexamethasone induced an increase in the β-AR receptor activity whereas the response to a combination of both agents was not additive. Thus, the impact of glucocorticoids and heregulin-1β alone and in combination on the β-AR activity needs to be examined more extensively.

In the current study, both heregulin-1β and leptin were shown to increase the rate of surfactant phospholipid synthesis in cultured fetal rat type II pneumocytes whereas only the former peptide was shown to increase the rate of phospholipid secretion from these cells. Heregulin-1β was also shown to induce higher β-AR activity in type II cells, which promoted a greater secretory response to (—)-isoproterenol. Given that leptin and heregulin-1β exert their effects via different receptors (Kirwin *et al.*, 2006; Zscheppang *et al.*, 2007), it would be of interest to investigate whether leptin is also capable of inducing β-AR activity and whether or not the effects of these two peptides on surfactant phospholipid synthesis are additive or synergistic.

4.5 Conclusions

In this study, a commercially available form of neuregulin-1β (heregulin-1β) has been shown to directly stimulate both the synthesis and secretion of surfactant phospholipids in cultured fetal rat type II pneumocytes. In addition, exposure of type II cells to this peptide enhanced the β-AR activity and, as a consequence, elevated the rate

of surfactant phospholipid secretion from these cells in response to the β-agonist (—)-isoproterenol. Although heregulin-1β had no effect on expression of the β-AR gene in type II cells exposed to the peptide alone, it was shown to act synergistically with dexamethasone in elevating the level of expression of the β-AR gene. All of these effects of neuregulin-1β, together with those of leptin and glucocorticoids, are summarized in Figure 4.1. The results for neuregulin-1β reported in this thesis therefore, not only support, but notably extend the proposal of Dammann *et al.* (2003) that neuregulin-1β plays an essential role in the differentiation and maturation of the lung in the later stages of gestation.

In order to understand the mechanism(s) by which neuregulin-1β exerts its numerous effects it needs to be recognized that, in both mice and rats, that the responses of lung cells to this peptide are mediated via ErbB receptors (Dammann *et al.*, 2006; Zscheppang *et al.*, 2006; Zscheppang *et al.*, 2007). Using a small interfering RNA (siRNA), which targeted the ErbB4 gene and silenced ErbB4 receptor activity in cultured 19-day fetal type II pneumocytes, Zscheppang *et al.* (2007) demonstrated that the resulting downregulation of the ErbB4 receptor caused a diminished rate of surfactant phospholipid synthesis. Their conclusion that the response of these cells to neuregulin-1β is mediated through ErbB4 receptor activation was supported by the observations that exposure of both fetal and adult rat lung epithelial cells to neuregulin-1β resulted in increased phosphorylation of the ErbB4 receptor (Liu *et al.*, 2007; Liu *et al.*, 2009).

Since neuregulin-1β (in this study heregulin-1β) has been shown to have a range of stimulatory effects on surfactant phospholipid synthesis and secretion, it could potentially be used as an alternative or supplementary treatment for overcoming the injurious effects of NRDS, which are common in prematurely delivered infants. The

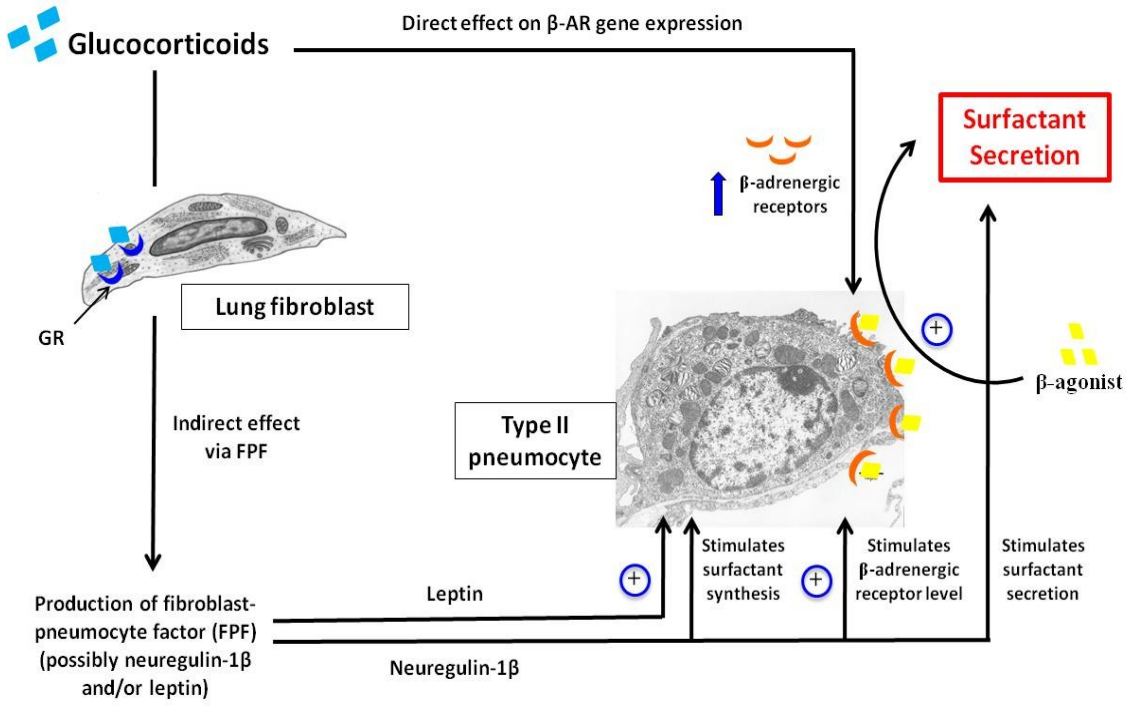

Figure 4.1 **Effects of glucocorticoids and neuregulin-1β on surfactant production by fetal type II pneumocytes**.

current regime for preventing the development of NRDS is the administration of glucocorticoids to pregnant women showing signs of imminent premature delivery (Liggins and Howie, 1972; Newnham and Moss, 2001). However, this procedure has two major drawbacks: (1) the stimulation of lung maturation by glucocorticoids is slow because it is an indirect effect and involves the production of FPF by lung fibroblasts (Smith, 1979; Post *et al.*, 1986; Smith and Post, 1989), and (2) male infants do not respond particularly well to this treatment (Torday *et al.*, 1981; Torday, 1984). In this context, neuregulin-1β administration would provide an improved method of treatment as it would act more rapidly through its direct action on the type II cells and the response to this peptide is the same in cells derived from both male and female fetuses. As the response times of type II cells to glucocorticoids and neuregulin-1β are dramatically different, it is suggested that a more effective treatment might involve the simultaneous administration of these two agents as it would provide a longer duration of response than either agent alone. However, it needs to be borne in mind that neuregulin-1β operates via ErbB receptors and that overexpression of members of this receptor family have been linked to both non-small cell lung and breast carcinomas (Brabender *et al.*, 2001; Yang *et al.*, 2006; Yang *et al.*, 2008; Pan *et al.*, 2011). Therefore, the concentration of neuregulin-1β used in the treatment of NRDS, as proposed above, would need to be carefully evaluated to ensure that none of these deleterious side effects are simultaneously activated. Such an approach would be consistent with the recommendation of Torday and Rehan (2007) that any physiologically or developmentally relevant interventions used in the treatment of lung diseases should be carefully selected.

References

Abraham, G., Kneuer, C., Ehrhardt, C., Honscha, W. & Ungemach, F. R. (2004). Expression of functional β2-adrenergic receptors in the lung epithelial cell lines 16HBE14o, Calu-3 and A549. *Biochimica et Biophysica Acta* **1691**, 169-179.

Aksoy, M. O., Mardini, I. A., Yang, Y., Zhou, S. & Kelsen, S. G. (2002). Glucocorticoid effects on the β-adrenergic receptor-adenylyl cyclase system of human airway epithelium. *Journal of Allergy and Clinical Immunology* **109**, 491-497.

Alangari, A. A. (2010). Genomic and non-genomic actions of glucocorticoids in asthma. *Annals of Thoracic medicine* **5**, 133-139.

Andreeva, A. V., Kutuzov, M. A. & Voyno-Yasenetskaya, T. A. (2007). Regulation of surfactant secretion in alveolar type II cells. *Am J Physiol Lung Cell Mol Physiol* **293**, L259-L271.

Asokananthan, N. & Cake, M. H. (1996). Stimulation of surfactant lipid secretion from fetal type II pneumocytes by gastrin-releasing peptide. *Am J Physiol Lung Cell Mol Physiol* **270**, L331-L337.

Avery, M. E. (2000). Surfactant deficiency in hyaline membrane disease: the story of discovery. *American Journal of Respiratory and Critical Care Medicine* **161**, 1074-1075.

Baselga, J. & Swain, S. M. (2009). Novel anticancer targets: Revisiting ERBB2 and discovering ERBB3. *Nature Reviews. Cancer* **9**, 463-475.

Bernhard, W., Raith, M., Pynn, C. J., Gille, C., Stichtenoth, G., Stoll, D., Schleicher, E. & Poets, C. F. (2011). Increased palmitoyl-myristoyl-phosphatidylcholine in neonatal rat surfactant is lung specific and correlates with oral myristic acid supply. *Journal of Applied Physiology* **111**, 449-457.

Besnard, V., Wert, S. E., Ikegami, M., Xu, Y., Heffner, C., Murray, S. A., Donahue, L. R. & Whitsett, J. A. (2011). Maternal synchronization of gestational length and lung maturation. *PLoS ONE* **6**, 1-10.

Bligh, E. G. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911-917.

Bock, A. V., Dill, D. B., Edwards, H. T., Henderson, L. J. & Talbott, J. H. (1929). On the partial ressures of oxygen and carbon dioxide in arterial blood and alveolar air. *Journal of Physiology* **68**, 277-291.

Brabender, J., Danenberg, K. D., Metzger, R., Schneider, P. M., Park, J. W., Salonga, D., Ho¨lscher, A. H. & Danenberg, P. V. (2001). Epidermal growth factor receptor and HER2-neu mRNA expression in non-small cell lung cancer is correlated with survival. *Clinical Cancer Research* **7**, 1850-1855.

Brehier, A. & Rooney, S. A. (1981). Phosphatidylcholine synthesis and glycogen depletion in fetal mouse lung: developmental changes and the effects of dexamethasone. *Experimental Lung Research* **2**, 273-287.

Brockes, J. P., Lemke, G. E. & Balzer DR, J. (1980). Purification and preliminary characterization of a glial growth factor from the bovine pituitary. *Journal of Biological Chemistry* **255**, 8374-8377.

Brown, L. A. S. & Longmore, W. J. (1981). Adrenergic and cholinergic regulation of lung surfactant secretion in the isolated perfused rat lung and in the alveolar type II cell in culture. *Journal of Biological Chemistry* **256**, 66-72.

Bruno, A., Pace, E., Chanez, P., Gras, D., Vachier, I., Chiappara, G., La Guardia, M., Gerbino, S., Profita, M. & Gjomarkaj, M. (2009). Leptin and leptin receptor expression in asthma. *Journal of Allergy and Clinical Immunology* **124**, 230-237.

Buckingham, S. & Avery, M. E. (1962). Time of appearance of lung surfactant in the fetal mouse. *Nature* **193**, 688-689.

Burden, S. & Yarden, Y. (1997). Neuregulins and their receptors: A versatile signaling module in organogenesis and oncogenesis. *Neuron* **18**, 847-855.

Busfield, S. J., Michnick, D. A., Chickering, T. W., Revett, T. L., Ma, J. Y., Woolf, E. A., Comrack, C. A., Dussault, B. J., Woolf, J., Goodearl, A. D. & Gearing, D. P. (1997). Characterization of a neuregulin-related gene, don-1, that is highly expressed in restricted regions of the cerebellum and hippocampus. *Molecular and Cellular Biology* **17**, 4007-4014.

Campbell, J. A. (1931). Gas tensions in the tissues *Physiological Reviews* **6**, 1-40.

Caniggia, I., Tseu, I., Han, R. N. N., Smith, B. T., Tanswell, K. & Post, M. (1991). Spatial and temporal differences in fibroblast behavior in fetal rat lung. *American Journal of Physiology* **261**, 424-433.

Cao, Z., Wu, X., Yen, L., Sweeney, C. & Carraway III, K. L. (2007). Neuregulin-induced ErbB3 downregulation is mediated by a protein stability cascade involving the E3 ubiquitin ligase Nrdp1. *Molecular and Cellular Biochemistry* **27**, 2180-2188.

Carey, M. A., Card, J. W., Voltz, J. W., Arbes Jr, S. J., Germolec, D. R., Korach, K. S. & Zeldin, D. C. (2007a). It's all about sex: gender, lung development and lung disease. *Am J Physiol Lung Cell Mol Physiol* **18**, L272-L278.

Carey, M. A., Card, J. W., Voltz, J. W., Germolec, D. R., Korach, K. S. & Zeldin, D. C. (2007b). The impact of sex and sex hormones on lung physiology and disease: lessons from animal studies. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **293**, L272-L278.

Carraway III, K. L., Weber, J. L., Unger, M. J., Ledesma, J., Yu, N., Gassmann, M. & Lai, C. (1997). Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. *Nature* **387**, 512-516.

Chailley-Heu, B., Chelly, N., Lelievre-Pegorier, M., Barlier-Mur, A. M., Merlet-Benichou, C. & Bourbon, J. R. (1999). Mild vitamin A deficiency delays fetal lung maturation in the rat. *American Journal of Respiratory Cell and Molecular Biology* **21**, 89-96.

Chatterjee, T. K., Liu, X., Davisson, R. L. & Fisher, R. A. (1997). Genomic organization of the rat pituitary adenylate cyclase-activating polypeptide receptor gene. *Journal of Biological Chemistry* **272**, 12122-12131.

Chelly, N., Mouhieddine-Gueddiche, O. B., Barlier-Mur, A. M., Chailly-Heu, B. & Bourbon, J. R. (1999). Keratinocyte growth factor enhances maturation of fetal rat lung type II cells *American Journal of Respiratory Cell and Molecular Biology* **20**, 423-432.

Cheng, J. B., Goldfien, A., Ballard, P. L. & Roberts, J. M. (1980). Glucocorticoids increase pulmonary β-adrenergic receptors in fetal rabbit. *Endocrinology* **107**, 1646-1648.

Cho, H. S., Mason, K., Ramyar, K. X., Stanley, A. M., Gabelli, S. B., Denney, D. W. & Leahy, D. J. (2003). Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* **421**, 756-760.

Citri, A., Kochupurakkal, B. S. & Yarden, Y. (2003). The deaf and the dumb: the biology of ErbB-2 and EbB-3. *Experimental Cell Research* **284**, 54-65.

Clements, J. A. & Avery, M. E. (1998). Lung surfactant and neonatal respiratory distress syndrome. *American Journal of Respiratory and Critical Care Medicine* **157**, S59-S66.

Clements, J. A., Brown, E. S. & Johnson, R. P. (1958). Pulmonary surface tension and the mucus lining of the lungs: some theoretical considerations. *Journal of Applied Physiology* **12**, 262-268.

Collins, S., Caron, M. G. & Lefkowitz, R. J. (1988). β2-Adrenergic receptors in hamster smooth muscle cells are transcriptionally regulated by glucocorticoids. *Journal of Biological Chemistry* **263**, 9067-9070.

Copenhaver, J. W. & Johnson, D. D. (1958). The respiratory system In *Bailey's Textbook of Histology*, pp. 393-414.

Copland, I. B. & Post, M. (2007). Stretch-activated signaling pathways responsible for early response gene expression in fetal lung epithelial cells. *Journal of Cellular Physiology* **210**, 133-143.

Copland, I. B., Reynaud, D., Pace-Asciak, C. & Post, M. (2006). Mechanotransduction of stretch- induced prostanoid release by fetal lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **291**, L487-L495.

Cornett, L. E., Hiller, F. C., Jacobi, S. E., Cao, W. & McGraw, D. W. (1998). Identification of a glucocorticoid response element in the rat β_2-adrenergic receptor gene. *Molecular Pharmacology* **54**, 1016-1023.

Creuwels, L. A. J. M., Van Golde, L. M. G. & Haagsman, H. P. (1997). The pulmonary surfactant system: Biochemical and clinical aspects. *Lung* **175**, 1-39.

Crouch, E. (1998). Collectins and pulmonary host defence. *American Journal of Respiratory Cell and Molecular Biology* **19** 177-210.

Crouch, E. & Wright, J. R. (2001). Surfactant proteins A and D and pulmonary host defence. *Annual Review of Physiology* **63**, 521-554.

Crovello, C. S., Lai, C., Cantley, L. C. & Carraway III, K. L. (1998). Differential signalling by the epidermal growth factor-like growth factors neuregulin-1 and neuregulin-2. *Journal of Biological Chemistry* **273**, 26954-26961.

Damas, J. E. (2010). The effect of serum, glucocorticoids, gender and gestational age on the activity of surfactant produced by cultured foetal rat type II pneumocytes. In *School of Biological Sciences and Biotechnology*, p. 222. Perth: Murdoch University.

Dammann, C. E. L., Nassimi, N., Liu, W. & Nielsen, H. C. (2006). ErbB receptor regulation by dexamethasone in mouse type II epithelial cells. *European Respiratory Journal* **28**, 1117-1123.

Dammann, C. E. L. & Nielsen, H. C. (1998). Regulation of the epidermal growth factor receptor in fetal rat lung fibroblasts during late gestation. *Endocrinology* **139**, 1671-1677.

Dammann, C. E. L., Nielsen, H. C. & Carraway III, K. L. (2003). Role of neuregulin-1β in the developing lung. *American Journal of Respiratory and Critical Care Medicine* **167**, 1711-1716.

Dammann, C. E. L., Ramadurai, S. M., McCants, D. D., Pham, L. D. & Nielsen, H. C. (2000). Androgen regulation of signaling pathways in late fetal mouse lung development. *Endocrinology* **141**, 2923-2929.

Dangel, V., Giray, J., Ratge, D. & Wisser, H. (1996). Regulation of β-adrenoceptor density and mRNA levels in the rat heart cell line H9c2. *Journal of Biochemistry* **317**, 925-931.

Davis, D. J., Dattel, B. J., Ballard, P. L. & Roberts, J. M. (1987). β-Adrenergic receptors and cyclic adenosine monophosphate generation in human fetal lung. *Pediatric Research* **21**, 142-147.

Deimling, J., Thompson, K., Tseu, I., Wang, J., Keijzer, R., Tanswell, A. K. & Post, M. (2007). Mesenchymal maintenance of distal epithelial cell phenotype during late fetal lung development. *Am J Physiol Lung Cell Mol Physiol* **292**, L725-L741.

Dhand, R., Sharma, V. K., Teng, A. L., Krishnasamy, S. & Gross, N. J. (1998). Protein-lipid interactions and enzyme requirements for light subtype generation on cycling reconstituted surfactant. *Biochemical and Biophysical Research Communications* **244**, 712-719.

Dobbs, L. G. (1990). Isolation and culture of alveolar type II cells. *Am J Physiol Lung Cell Mol Physiol* **258**, L134-L147.

Dobbs, L. G., Gonzalez, R. F. & Williams, M. C. (1986). An improved method for isolating type II cells in high yield and purity. *American Review of Respiratory Disease* **134**, 141-145.

Dobbs, L. G. & Mason, R. J. (1979). Pulmonary alveolar type II cells isolated from rats: Release of phosphatidylcholine in response to β-adrenergic stimulation. *Journal of Clinical Investigation* **63**, 378-387.

Dorn, C. C., Rice, W. R. & Singleton, F. M. (1989). Calcium mobilization and response recovery following P2-purinoceptor stimulation of rat isolated alveolar type II cells. *British Journal of Pharmacology* **97**, 163-170.

Douglas, W. H. J. & Farrell, P. M. (1976). Isolation of cells that retain differentiated functions *in vitro:* Properties of clonally isolated type II alveolar pneumonocytes *Environmental Health Perspectives* **16**, 83-88.

Emanuel, R. L., Torday, J. S., Mu, Q., Asokananthan, N., Sikorski, K. A. & Sunday, M. E. (1999). Bombesin-like peptides and receptors in normal fetal baboon lung: roles in lung growth and maturation *Am J Physiol Lung Cell Mol Physiol* **277**, L1003-L1017.

Emorine, L. J., Marullo, S., Delavier-Klutchko, C., Kaveri, S. V., Durieu-Trautmann, O. & Strosberg, A. D. (1987). Structure of the gene for human β_2-adrenergic receptor: Expression and promoter characterization. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 6995-6999.

Enhorning, G. & Holm, B. A. (1993). Disruption of pulmonary surfactant's ability to maintain openness of a narrow tube. *Journal of Applied Physiology* **74**, 2922-2927.

Esper, R. M. & Loeb, J. A. (2009). Neurotrophins induce neuregulin release through protein kinase cactivation. *Journal of Biological Chemistry* **284**, 26251-26260.

Fabisiak, J. P., Vesell, E. S. & Rannels, D. E. (1987). Interactions of beta adrenergic antagonists with isolated rat alveolar type II pneumocytes. I. Analysis, characterization and regulation of specific beta adrenergic receptors. *Journal of Pharmacology and Experimental Therapeutics* **241**, 722-727.

Falls, D. L. (2003). Neuregulin: functions, forms, and signalling strategies. *Experimental Cell Research* **284**, 14-30.

Falls, D. L., Harris, D. A., Johnson, F. A., Morgan, M. M., Corfas, G. & Fischbach, G. D. (1990). Mr 42,000 ARIA: A protein that may regulate the accumulation of acetylcholine receptors at developing chick neuromuscular junctions. *Cold Spring Harbor Symposia on Quantitative Biology* **55**, 397-406.

Floros, J., Nielsen, H. C. & Torday, J. S. (1987). Dihydrotestosterone blocks fetal lung fibroblast-pneumocyte factor at a pretranslational level. *Journal of Biological Chemistry* **262**, 13592-13598.

Fraslon, C. & Bourbon, J. R. (1992). Comparison of effects of epidermal and insulin-like growth factors, gastrin releasing peptide and retinoic acid on fetal lung cell growth and maturation in vitro. *Biochimica et Biophysica Acta* **1123**, 65-75.

Fraslon, C. & Bourbon, J. R. (1994). Retinoids control surfactant phospholipid biosynthesis in fetal rat lung. *Am J Physiol Lung Cell Mol Physiol* **266**, L705-L712.

Geshe, j., Fehrenbach, H., Koslowski, R., Ohler, F. M., Pynn, C. J., Griese, M., Poets, C. F. & Bernhard, W. (2011). rhKGF stimulates lung surfactant production in neonatal rats *in vivo*. *Pediatric Pulmonology* **46**, 882-895.

Giannopoulos, G. & Sommers Smith, S. K. (1982). Hormonal regulation of β-adrenergic receptors in fetal rabbit lung in organ culture. *Life Sciences* **31**, 795-802.

Glasser, S. W., Burhans, M. S., Korfhagen, T. R., Na, C. L., Sly, P. D., Ross, G. F., Ikegami, M. & Whitsett, J. A. (2001). Altered stability of pulmonary surfactant in SP-C deficient mice. *PNAS* **98**, 6366-6371.

Gluck, L., Motoyama, E. K., Smits, H. L. & Kulovich, M. V. (1967). The biochemical development of surface activity in mammalian lung. I. The surface-active phospholipids; the separation and distribution of surface-active lecithin in the lung of the developing rabbit fetus. *Pediatric Research* **1**, 237-246.

Griese, M. (1999). Pulmonary surfactant in health and human lung disease. *European Respiratory Journal* **13**, 1455-1476.

Haagsman, H. P. & Van Golde, L. M. G. (1991). Synthesis and assembly of lung surfactant. *Annual Review of Physiology* **53**, 441-464.

Hadcock, J. R. & Malbon, C. C. (1988). Regulation of β-adrenergic receptors by "permissive" hormones: Glucocorticoids increase steady-state levels of receptor mRNA. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 8415-8419.

Hadcock, J. R., Wang, H. & Malbon, C. (1989). Agonist-induced destabilization of β-adrenergic receptor mRNA attenuation of glucocorticoid-induced up-regulation of β-adrenergic receptor *Journal of Biological Chemistry* **264**, 19928-19933.

Haies, D. M., Gil, J. & Weibel, E. R. (1980). Morphometric study of rat lung cells. *American Review of Respiratory Disease* **123**, 533-541.

Hallman, M., Enhorning, G. & Possmayer, F. (1985). Composition and surface activity of normal and phosphatidylglycerol-deficient lung surfactant. *Pediatric Research* **19**, 286-292.

Hallman, M., Feldman, B. H., Kirkpatrick, E. & Gluck, L. (1977). Absence of phosphatidylglycerol (PG) in respiratory distress syndrome in the newborn. *Pediatric Research* **11**, 714-720.

Hallman, M. & Gluck, L. (1976). Phosphatidylglycerol in lung surfactant: III. possible modifier of surfactant function. *Journal of Lipid Research* **17**, 257-262.

Hallman, M. & Haataja, R. (2007). Genetic basis of respiratory distress syndrome. *Frontiers in Bioscience* **12**, 2670-2682.

Harari, D., Tzahar, E., Romano, J., Shelly, M., Pierce, J. H., Andrews, G. C. & Yarden, Y. (1999). Neuregulin-4: A novel growth factor that acts through the ErB-4 receptor tyrosine kinase. *Oncogene* **18**, 2681-2689.

Hinegardner, R. T. (1971). An improved fluorometric assay for DNA. *Analytical Biochemistry* **39**, 197-201.

Hoggard, N., Hunter, L., Duncan, J. S., Williams, L. M., Trayhurn, P. & Mercer, J. G. (1997). Leptin and leptin receptor mRNA and protein expression in the murine fetus and placenta. *PNAS* **94**, 11073-11078.

Holm, B. A., Wang, Z., Egan, E. A. & Notter, R. H. (1996). Content of dipalmitoyl phosphatidylcholine in lung surfactant: Ramifications for surface activity. *Pediatric Research* **39**, 805-811.

Holmes, W. E., Sliwkowksi, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W. J., Wood, W. I., Goeddel, D. V. & Vandlen, R. L. (1992). Identification of heregulin: A specific activator of p185 erbB2. *Science* **256**, 1205-1210.

Holmskov, U., Lawson, P., Teisner, B., Tornøe, I., Willis, A. C., Morgani, C., Koch, C. & Reid, K. B. M. (1997). Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein-D binding molecule. *Journal of Biological Chemistry* **272**, 13743–13749.

Huang, Z., Wang, W., Nayak, P. S., Dammann, C. E. & Sanchez-Esteban, J. (2012). Stretch-induced fetal type II cell differentiation is mediated via ErbB1-ErbB4 interactions. *Journal of Biological Chemistry* **287**, 18091-18102.

Jobe, A. H. (1993). Pulmonary surfactant therapy *New England Journal of Medicine* **328**, 861-868.

Johannson, J. & Curstedt, T. (1997). Molecular structure and interactions of pulmonary surfactant components. *European Journal of Biochemistry* **244**, 675-693.

Kevill, K. A., Bhandari, V., Kettunen, M., Leng, L., Fan, J., Mizue, Y., Dzuira, J. D., Reyes-Mugica, M., McDonald, C. L., Baugh, J. A., O'Connor, C. L., Aghai, Z. H., Donnelly, S. C., Bazzy-Asaad, A. & Bucala II, R. J. (2008). A role for macrophage migration inhibitory factor in the neonatal respiratory distress syndrome. *Journal of Immunology* **180**, 601-608.

Kikkawa, Y. & Yoneda, K. (1974). The type II epithelial cell of the lung. *Laboratory Investigation* **30**, 76-84.

King, R. J. (1982). Pulmonary surfactant. *Journal of Applied Physiology* **53**, 1-8.

King, R. J., Carmichael, C. & Horowitz, P. M. (1983). Reassembly of lipid-protein complexes of pulmonary surfactant: proposed mechanism of interaction. *Journal of Biological Chemistry* **258**, 10672-10680.

King, R. J., Klass, D. J. & Clements, J. A. (1973). Isolation of apoproteins from canine surface active material. *American Journal of Physiology* **224**, 789-795.

King, R. J. & Martin, H. (1980). Intracellular metabolism of the apoproteins of pulmonary surfactant in rat lung. *Journal of Applied Physiology* **48**, 812-820.

Kirwin, S. M., Bhandari, V., Dimatteo, D., Barone, C., Johnson, L., Saptarashi, P., Spitzer, A. R., Chander, A., Hassink, S. G. & Funanage, V. L. (2006). Leptin enhances lung maturation in the fetal rat. *Pediatric Research* **60**, 200-204.

Klaus, M. H., Clements, J. A. & Havel, R. J. (1961). Composition of surface-active material isolated from beef lung. *PNAS* **47**, 1858-1859.

Kobilka, B. K., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Dixon, R. A. F., Keller, P., Caron, M. G. & Lefkowitz, R. J. (1987). Delineation of the intronless nature of the genes for the human and hamster β_2-adrenergic receptor and their putative promoter regions *Journal of Biological Chemistry* **262**, 7321-7327.

Kotas, R. V. & Avery, M. E. (1971). Accelerated appearance of pulmonary surfactant in the fetal rabbit. *Journal of Applied Physiology* **30**, 358-361.

Lang, C. J., Postle, A. D., Orgeig, S., Possmayer, F., Bernhard, W., Panda, A. K., Jurgens, K. D., Milsom, W. K., Nag, K. & Daniels, C. B. (2005). Dipalmitoylphosphatidylcholine is not the major surfactant phospholipid species in all mammals. *American Journal of Physiology* **289**, R1426-R1439.

Lawson, E. E., Birdwell, R. L., Huang, P. S. & Taeusch, H. W. J. (1979). Augmentation of pulmonary surfactant secretion by lung expansion at birth. *Pediatric Research* **13**, 611-614.

Ledford, J. G., Goto, H., Potts, E. N., Degan, S., Chu, H. W., Voelker, D. R., Sunday, M. E., Cianciolo, G. J., Foster, W. M., Kraft, M. & Wright, J. R. (2009). SP-A preserves airway homeostasis during mycoplasma pneumoniae Infection in mice. *Journal of Immunology* **182**, 7818-7827.

Lefkowitz, R. J., Mukherjee, C., Coverstone, M. & Caron, M. G. (1974). Stereospecific [^{3}H] (-)-alprenolol binding sites, β-adrenergic receptors and adenylate cyclase. *Biochemical and Biophysical Research Communications* **60**, 703-709.

Lemke, G. (1996). Neuregulins in development *Molecular and Cellular Neurosciences* **7**, 247-262.

Leung, J. C. K., Chan, L. Y. Y., Tang, S. C. W., Chu, K. M. & Lai, K. N. (2006). Leptin induces TGF-β synthesis through functional leptin receptor expressed by human peritoneal mesothelial cell. *Kidney International* **69**, 2078-2086.

Liggins, G. C. (1969). Premature delivery of foetal lambs infused with glucocorticoids *Journal of Endocrinology* **45**, 515-523.

Liggins, G. C. & Howie, R. N. (1972). A controlled trial of antepartum glucocorticoid treatment for preventation of the respiratory distress syndrome in premature infants. *Pediatrics* **50**, 515-525.

Lin, R., Degan, S., Theriot, B. S., Fischer, B. M., Strachan, R. T., Liang, J., Pierce, R. A., Sunday, M. E., Noble, P. W., Kraft, M., Brody, A. R. & Walker, J. K. L. (2012). Chronic treatment in vivo with β-adrenoceptor agonists induces dysfunction of airway β2-adrenoceptors and exacerbates lung inflammation in mice. *British Journal of Pharmacology* **165**, 2365-2377.

Liu, M., Wang, L., Li, E. & Enhorning, G. (1991). Pulmonary surfactant will secure free airflow through a narrow tube. *Journal of Applied Physiology* **71** 742-748.

Liu, W., Purevdorj, E., Zscheppang, K., Von Mayersbach, D., Behrens, J., Brinkhaus, M. J., Nielsen, H. C., Schmiedl, A. & Dammann, C. E. (2010). ErbB4 regulates the timely progression of late fetal lung development. *Biochimica et Biophysica Acta* **1803**, 832-839.

Liu, W., Volpe, M. V., Zscheppang, K., Nielsen, H. C. & Dammann, C. E. L. (2009). ErbB4 regulates surfactant synthesis and proliferation in adult rat pulmonary epithelial cells. *Experimental Lung Research* **35**, 29-47.

Liu, W., Zscheppang, K., Murray, S., Nielsen, H. C. & Dammann, C. E. L. (2007). The ErbB4 receptor in fetal rat lung fibroblasts and epithelial type II cells. *Biochimica et Biophysica Acta* **1772**, 737-747.

Mak, J. C. W., Nishikawa, M. & Barnes, P. J. (1995a). Glucocorticosteroids increase β_2-adrenergic receptor transcription in human lung. *Am J Physiol Lung Cell Mol Physiol* **268**, L41-L46.

Mak, J. C. W., Nishikawa, M., Shirasaki, H., Miyayasu, K. & Barnes, P. J. (1995b). Protective effects of glucocorticoid on downregulation of pulmonary β2-adrenergic receptors in vivo. *Journal of Clinical Investigation* **96**, 99-106.

Maker, G. L. (2008). Regulation of surfactant production by fetal type II pneumocytes and the characterization of fibroblast-pneumocyte factor. In *School of Biological Sciences and Biotechnology*, p. 175. Perth, Western Australia Murdoch University.

Mallory Jr, G. B. (2001). Surfactant proteins: role in lung physiology and disease in early life *Pediatric Respiratory Review* **2**, 151-158.

Maniscalco, W. M. & Shapiro, D. L. (1983). Effects of dexamethasone on β-adrenergic receptors in fetal lung explants. *Pediatric Research* **17**, 274-277.

Marchionni, M. A., Goodearl, A. D., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F. J., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J. B., Hsuan, J. J., Totty, N. F., Otsu, M., McBurney, R. N., Waterfield, M. D., Stroobant, P. & Gwynne, D. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* **362**, 312-318.

Massaro, D. & Massaro, G. D. (2006). Estrogen receptor regulation of pulmonary alveolar dimensions: alveolar sexual dimorphism in mice. *American Journal of Physiology. Lung Cellular and Molecular Physiology* **290**, L866-L870.

Mautino, B., Costa, L. D., Gambarotta, G., Perroteau, I., Fasolo, A. & Datia, C. (2004). Bioactive recombinant neuregulin-1, -2, and -3 expressed in *Escherichia coli*. *Protein Expression and Purification* **35**, 25-31.

McDevitt, T. M., Gonzales, L. W., Savani, R. C. & Ballard, P. L. (2007). Role of endogenous TGF-β in glucocorticoid-induced lung type II cell differentiation. *Am J Physiol Lung Cell Mol Physiol* **292**, L249-L257.

McGraw, D. W., Chai, S. E., Hiller, F. C. & Cornett, L. E. (1995). Regulation of the β_2-adrenergic receptor and its mRNA in the rat lung by dexamethasone. *Experimental Lung Research* **21**, 535-546.

Miller, H. C. & Futrakul, P. (1968). Birth weight, gestational age, and sex as determining factors in the incidence of respiratory distress syndrome of prematurely born infants. *Journal of Pediatrics* **72**, 628-635.

Muglia, L., Jacobson, L., Dikkes, P. & Majzoub, J. (1995). Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need *Nature* **373**, 427-432.

Muglia, L. J., Bae, D. S., Brown, T. T., Vogt, S. K., Alvarez, J. G., Sunday, M. E. & Majzoub, J. A. (1999). Proliferation and differentiation defects during lung development in corticotropin-releasing hormone-deficient mice. *American Journal of Respiratory Cell and Molecular Biology* **20**, 181-188.

Mutlu, G. M. & Factor, P. (2008). Alveolar epithelial β2-adrenergic receptors. *American Journal of Respiratory Cell and Molecular Biology* **38**, 127-134.

Newnham, J. P. & Moss, T. J. (2001). Antenatal glucocorticoids and growth: single versus multiple doses in animal and human studies. *Seminars in Neonatology* **6**, 285-292.

Nielsen, H. C. (1989). Epidermal growth factor influences the developmental clock regulating maturation of the fetal lung fibroblast . *Biochimica et Biophysica Acta* **1012**, 201-206.

Nielsen, H. C. & Torday, J. S. (1981). Sex differences in fetal rabbit pulmonary surfactant production. *Pediatric Research* **15**, 1245-1247.

Nielsen, H. C. & Torday, J. S. (1983). Anatomy of fetal rabbit gonads and the sexing of fetal rabbits. *Laboratory Animals* **17**, 148-150.

Nielsen, H. C., Zinman, H. M. & Torday, J. S. (1982). Dihydrotestosterone inhibits fetal rabbit pulmonary surfactant production *The Journal of Clinical Investigation* **69**, 611-616.

Norris, J. S., Brown, P., Cohen, J., Cornett, L. E., Kohler, P. O., MacLeod, S. L., Popovick, K., Robey, R. B., Sifford, M., Syms, A. J. & Smith, R. G. (1987). Glucocorticoid induction of β-adrenergic receptors in the DDT1 MF-2 smooth muscle cell line involves synthesis of new receptor. *Molecular and Cellular Biochemistry* **74**, 21-27.

Ormond, O. J., Orgeig, S., Daniels, C. B. & Milsom, W. K. (2003). Thermal acclimation of surfactant secretion and its regulation by adrenergic and cholinergic agonists in type II cells isolated from warm-active and torpid goldenmantled ground squirrels, *Spermophilus lateralis*. *Journal of Experimental Biology* **206**, 3031-3041.

Pan, M., Lin, Y., Lin, C., Wei, C., Ho, C. & Chen, W. (2011). Suppression of heregulin-β1/HER2-modulated invasive and aggressive phenotype of breast carcinoma by pterostilbene via inhibition of matrix metalloproteinase-9, p38 kinase cascade and Akt activation. *Evidence-based Complementary and Alternative Medicine* **2009**, 1-12.

Pattle, R. E. (1955). Properties, function and origin of the alveolar lining layer. *Nature* **175**, 1125-1126.

Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben Levy, R. & Yarden, Y. (1992). Isolation of the Neu/HER-2 stimulatory ligand: a 44kDa glycoprotein that induces differentiation of mammary tumor cells. *Cell* **69**, 205-216.

Persson, A., Chang, D., Rust, K., Moxley, M., Longmore, W. & Crouch, E. (1989). Purification and biochemical characterization of CP4 (SP-D): a collagenous surfactant-associated protein. *Biochemistry* **28**, 6361–6367.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, 2002-2007.

Post, M., Barsoumian, A. & Smith, B. T. (1986). The cellular mechanism of glucocorticoid acceleration of fetal lung maturation. *Journal of Biological Chemistry* **261**, 2179-2184.

Post, M., Floros, J. & Smith, B. T. (1984). Inhibition of lung maturation by monoclonal antibodies against fibroblast-pneumocyte factor. *Nature* **308**, 284-286.

Post, M. & Smith, B. T. (1984). Effect of fibroblast-pneumocyte factor on the synthesis of surfactant phospholipids in type II cells from fetal rat lung. *Biochimica et Biophysica Acta* **793**, 297-299.

Provost, P. R., Boucher, E. & Tremblay, Y. (2009). Apolipoprotein A-I, A-II, C-II, and H expression in the developing lung and sex difference in surfactant lipids. *Journal of Endocrinology* **200**, 321-330.

Rice, W. R. & Singleton, F. M. (1987). P2Y-purinoceptor regulation of surfactant secretion from rat isolated alveolar type II cells is associated with mobilization of intracellular calcium. *British Journal of Pharmacology* **91**, 833-838.

Ridsdale, R. & Post, M. (2004). Surfactant lipid synthesis and lamellar body formation in glycogen-laden type II cells. *Am J Physiol Lung Cell Mol Physiol* **287** L743-L751.

Riese, D. J., van Raaij, T. M., Plowman, G. D., Andrews, G. C. & Stern, D. F. (1995). The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Molecular and Cellular Biology* **15**, 5770-5776.

Rooney, S. A. (2001). Regulation of surfactant secretion. *Comparative Biochemistry and Physiology* **129**, 233-243.

Rooney, S. A., Young, S. L. & Mendelson, C. R. (1994). Molecular and cellular processing of lung surfactant. *FASEB Journal* **8** 957-967.

Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S. & Aaronson, S. A. (1989). Purification and characterisation of a newly identified growth factor specific for epithelial cells. *Biochemistry* **86**, 802-806.

Rubin, L. P., Kovacs, C. S., De Paepe, M. E., Tsai, S. W., Torday, J. S. & Kronenberg, H. M. (2004). Arrested pulmonary alveolar cytodifferentiation and defective surfactant synthesis in mice missing the gene for parathyroid hormone-related protein. *Developmental Dynamics* **230**, 278-289.

Rugg, E. L., Barnett, D. B. & Nahorski, S. R. (1978). Coexistence of $beta_1$ and $beta_2$ adrenoceptors in mammalian lung: Evidence from direct binding studies *Molecular Pharmacology* **14**, 996-2005.

Samtani, M. N., Pyszczynski, N. A., DuBois, D. C., Almon, R. R. & Jusko, W. J. (2006). Modeling glucocorticoid-mediated fetal lung maturation: II. Temporal patterns of gene expression in fetal rat lung. *Journal of Pharmacology and Experimental Therapeutics* **317**, 127-138.

Schmitz, G. & Muller, G. (1991). Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. *Journal of Lipid Research* **32**, 1539-1570.

Schwartz, R. M., Luby, A. M., Scanlon, J. W. & Kellogg, R. J. (1994). Effect of surfactant on morbidity, mortality,and resource use in newborn infants weighing 500 to 1500 g. *New England Journal of Medicine* **330**, 476–1480.

Sen, N. (1991). Hormonal regulation of surfactant production in cultured fetal lung cells In *School of Biological Sciences and Biotechnology*, p. 170. Perth, Western Australia Murdoch University

Sen, N. & Cake, M. H. (1991). Enhancement of disaturated phosphatidylcholine synthesis by epidermal growth factor in cultured fetal lung cells involves a fibroblast-epithelial cell interaction. *American Journal of Respiratory Cell and Molecular Biology* **5**, 337-343.

Shan, L., Emanuel, R. L., Dewald, D., Torday, J. S., Asokanathan, N., Wada, K., Wada, E. & Sunday, M. E. (2004). Bombesin-like peptide receptor gene expression, regulation, and function in fetal murine lung. *Am J Physiol Lung Cell Mol Physiol* **286**, L165-L173.

Shi, M., Liu, D., Duan, H., Qian, L., Wang, L., Niu, L., Zhang, H., Yong, Z., Gong, Z., Song, L., Yu, M., Hu, M., Xia, Q., Shen, B. & Guo, N. (2011). The β2-adrenergic receptor and Her2 comprise a positive feedback loop in human breast cancer cells. *Breast Cancer Research and Treatment* **125**, 351-362.

Slieker, L. J., Sloop, K. W., Surface, P. L., Kriauciunas, A., LaQuier, F., Manetta, J., Bue-Valleskey, J. & Stephens, T. W. (1995). Regulation of expression of ob mRNA and protein by glucocorticoids and cAMP. *Journal of Biological Chemistry* **271**, 5301-5304.

Smith, B. T. (1978). Fibroblast-pneumonocyte factor: intercellular mediator of glucocorticoid effect on fetal lung. In *Intensive Care in the Newborn II* (L. Stern, W. O., and B. Friis-Hansen, ed.), pp. 25-32. New York Masson.

Smith, B. T. (1979). Lung maturation in the fetal rat: accelaration by injection of fibroblast-pneumocyte factor. *Science* **204**, 1094-1095.

Smith, B. T. & Fletcher, W. A. (1979). Pulmonary epithelial-mesenchymal interactions: Beyond organogenesis. *Human Pathology* **10**, 248-250.

Smith, B. T. & Post, M. (1989). Fibroblast-pneumocyte factor. *Am J Physiol Lung Cell Mol Physiol* **257**, L174-L178.

Smith, B. T. & Sabry, K. (1983). Glucocorticoid-thyroid synergism in lung maturation: a mechanism involving epithelial-mesenchymal interaction. *Developmental Biology* **80**, 1951-1954.

Snyder, J. M., Johnston, J. M. & Mendelson, C. R. (1981). Differentiation of type II cells of human fetal lung in vitro. *Cell and Tissue Research* **220**, 17-25.

Stahlman, M. T., Gray, M. P., Falconieri, M. W., Whitsett, J. A. & Weaver, T. E. (2000). Lamellar body formation in normal and surfactant protein B-deficient fetal mice. *Laboratory Investigation* **80**, 395-403.

Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., Brynjolfsson, J., Gunnarsdottir, S., Ivarsson, O., Chou, T. T., Hjaltason, O., Birgisdottir, B., Jonsson, H., Gudnadottir, V. G., Gudmunsdottir, E., Bjornsson, A., Ingvarsson, B., Ingason, A., Sigfusson, S., Hardardottir, H., Harvey, R. P., Lai, D., Zhou, M., Brunner, D., Mutel, V., Gonzalo, A., Lemke, G., Sainz, J., Johanesson, G., Andresson, T., Gudbjartsson, D., Manolescu, A., Frigge, M. L., Gurney, M. E., Kong, A., Gulcher, J. R., Petursson, H. & Stefansson, K. (2002). *Neuregulin 1* and susceptibility to shizophrenia. *American Journal of Human Genetics* **71**, 877-892.

Strader, C. D., Fong, T. M., Graziano, M. P. & Tota, M. R. (1995). The family of G protein coupled receptors. *FASEB Journal* **9**, 745-754.

Sunday, M. E., Hua, J., Dai, H. B., Nusrat, A. & Torday, J. S. (1990). Bombesin increases fetal lung growth and maturation *in utero* and in organ culture. *American Journal of Respiratory Cell and Molecular Biology* **3**, 199-205.

Swenson, E. R., Robertson, T., Polissar, N. L., Middaugh, M. E. & Hlastala, M. P. (1992). Conducting airway gas exchange: diffusion-related differences in inert gas elimination *Journal of Applied Physiology* **72**, 1581-1588.

Thannhauser, S. J., Benotti, J. & Boncoddo, N. F. (1946). Isolation and properties of hydrolecithin (dipalmityllecithin) from lung; its occurrence in the sphinogomyelin fraction of animal tissues. *Journal of Biochemistry* **166**, 669–675.

Togari, A., Arai, M., Mizutani, S., Mizutani, S., Koshihara, Y. & Nagatsu, T. (1997). Expression of mRNAs for neuropeptide receptors and β-adrenergic receptors in human osteoblasts and human osteogenic sarcoma cells. *Neuroscience Letters* **233**, 125-128.

Togaria, A., Araia, M., Mizutania, S., Mizutania, S., Koshiharab, Y. & Nagatsuc, T. (1997). Expression of mRNAs for neuropeptide receptors and β-adrenergic receptors in human osteoblasts and human osteogenic sarcoma cells. *Neuroscience Letters* **233**, 125-128.

Torday, J. S. (1984). The sex difference in type II cell surfactant synthesis originates in the fibroblast in vitro. *Experimental Lung Research* **7**, 187-194.

Torday, J. S., Ihida-Stansbury, K. & Rehanc, V. K. (2009). Leptin stimulates Xenopus lung development: evolution in a dish. *Evolution and Development* **11**, 218-223.

Torday, J. S. & Kourembanas, S. (1990). Fetal rat lung fibroblasts produce a TGFβ homolog that blocks alveolar type II cell maturation. *Developmental Biology* **139**, 35-41.

Torday, J. S., Nielsen, H. C., Fencl, M. & Avery, M. E. (1981). Sex differences in fetal lung maturation. *American Review of Respiratory Disease* **123**, 205-208.

Torday, J. S. & Rehan, V. K. (2002). Stretch-stimulated surfactant synthesis is coordinated by the paracrine actions of PTHrP and leptin. *Am J Physiol Lung Cell Mol Physiol* **283**, L130-L135.

Torday, J. S. & Rehan, V. K. (2006). Up-regulation of fetal rat lung parathyroid hormone-related protein gene regulatory network down-regulates the sonic hedgehog/wnt/ βcatenin gene regulatory network. *Pediatric Research* **60**, 382-388.

Torday, J. S. & Rehan, V. K. (2007). Developmental cell/molecular biologic approach to the etiology and treatment of bronchopulmonary dysplasia. *Pediatric Research* **62**, 2-7.

Torday, J. S., Sun, H., Wang, L., Torres, E., Sunday, M. E. & Rubin, L. P. (2002). Leptin mediates the parathyroid hormone-related protein (PTHrP) paracrine stimulation of fetal lung maturation. *Am J Physiol Lung Cell Mol Physiol* **282**, L405-L410.

Van der Velden, V. H. J. (1998). Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma. *Mediators of Inflammation* **7**, 229-237.

Vermeer, P. D., Einwalter, L. A., Moninger, T. O., Rokhlina, T., Kern, J. A., Zabner, J. & Welsh, M. J. (2003). Segregation of receptor and ligand regulates activation of epithelial growth factor receptor. *Nature* **422**, 322-326.

von Ende, C. N. (2001). Repeated measures analysis: growth and other time-dependent measures. In *Design and analysis of ecological experiments, 2nd edition* (Scheiner, S. M. & Gurevitch, J., eds.), pp. 134-157. New York: Oxford University Press

Weaver, T. E. & Conkright, J. J. (2001). Function of surfactant proteins B and C. *Annual Review of Physiology* **63**, 555-578.

Whitsett, J. A., Manton, M. A., Darovec-Beckerman, C. & Adams, K. (1981). Catecholamines and their receptors in the developing rat lung. II. β-adrenergic receptors and catecholamine sensitive adenylate cyclase in the developing rat lung *Life Sciences* **28**, 339-345.

Whitsett, J. A. & Weaver, T. E. (2002). Hydrophobic surfactant proteins in lung function and disease. *New England Journal of Medicine* **347**, 2141-2148.

Wirtz, H. R. W. & Dobbs, L. G. (1990). Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells. *Science* **250**, 1266-1269.

Wright, J. R. (1990). Clearance and recycling of pulmonary surfactant. *Am J Physiol Lung Cell Mol Physiol* **259**, L1-L12.

Wright, J. R. (1997). Immunomodulatory functions of surfactant. *Physiological Reviews* **74**, 931-962.

Xu, X., McCormick-Shannon, K., Voelker, D. R. & Mason, R. J. (1998). KGF increases SP-A and SP-D mRNA levels and secretion in cultured rat alveolar type II cells *American Journal of Respiratory Cell and Molecular Biology* **18**, 168-178.

Yang, C., Klein, E. A., Assoian, R. K. & Kazanietz, M. G. (2008). Heregulin β1 promotes breast cancer cell proliferation through Rac/ERK-dependent induction of cyclin D1 and p21Cip1. *Biochemical Journal* **410**, 167-175.

Yang, C., Liu, Y., Lemmon, M. A. & Kazanietz, M. G. (2006). Essential role for Rac in heregulin-β1 mitogenic signaling: a mechanism that involves epidermal growth factor receptor and is independent of ErbB4. *Molecular and Cellular Biology* **26**, 831-842.

Yu, S. H. & Possmayer, F. (1988). Comparative studies on the biophysical activities of low-molecular-weight hydrophobic proteins purified from bovine pulmonary surfactant. *Biochemical et Biophysical Acta* **961**, 337-350.

Zhang, D., Sliwkowksi, M. X., Mark, M., Frantz, G., Akita, R. W., Sun, Y., Hillan, K., Crowley, C., Brush, J. & Godowski, P. J. (1997). Neuregulin-3 (NRG3): A novel neural tissue-enriched protein that binds and activates ErbB4. *PNAS* **94**, 9562-9567.

Zhang, J. P., Wang, Y. L., Wang, Y. H., Zhang, R., Chen, H. & Su, H. B. (2004). Prophylaxis of neonatal respiratory distress syndrome by intra-amniotic administration of pulmonary surfactant. *Chinese Medical Journal* **117**, 120-124.

Zhao, Y. Y., Sawyer, D. R., Baliga, R. R., Opel, D. J., Han, X., Marchionni, M. A. & Kelly, R. A. (1998). Neuregulins promote survival and growth of cardiac myocytes persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. *Journal of Biological Chemistry* **273**, 10261-10269.

Zscheppang, K., Korenbaum, E., Bueter, W., Ramadurai, S. M., Nielsen, H. C. & Dammann, C. E. L. (2006). ErbB receptor dimerization, localization, and co-localization in mouse lung type II epithelial cells. *Pediatric Pulmonology* **41**, 1205-1212.

Zscheppang, K., Liu, W., Volpe, M. V., Nielsen, H. C. & Dammann, C. E. L. (2007). ErbB4 regulates fetal surfactant phospholipid synthesis in primary fetal rat type II cells. *Am J Physiol Lung Cell Mol Physiol* **293**, L429-L435.